DETERMINATION OF THE FUNCTION OF AUTOANTIBODIES IN THE NOD MOUSE MODEL OF SJOGREN'S SYNDROME

By

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The NOD mouse develops a condition similar to the human disease, Sjögren's syndrome (SS), an autoimmune disease primarily affecting the salivary and lachrymal glands. The disease manifests as oral and ocular dryness and is further associated with focal lymphocytic infiltration of the glands and the development of serum autoantibodies. The primary goal of this dissertation is to explore the role of the autoantibodies in the disease pathogenesis.

In vivo studies focused on 3 congenic strains of the NOD mouse. The NOD.Igμ^{null} mouse lacks specifically B cells, while the NOD.IL-4$^{-/-}$ and NOD.IFN-$\gamma^{-/-}$ mouse strains

lack specific cytokines important in the effective generation of the humoral and inflammatory adaptive immune responses. The B cell knockout mouse demonstrates that B cells are critical for the loss of exocrine function. Furthermore, passive transfer studies demonstrate that SS patients' IgG can induce secretory hypofunction in these mice. The combination of these three mouse strains help to dissect the role of the autoantibodies and other aspects of the disease contributing to secretory dysfunction. In these mice, elevated apoptotic activity can be detected in all strains with the exception of the IFN-$\gamma^{-/-}$ although none lose secretory function. This suggests that apoptosis alone is not sufficient for loss of function. Increased inflammation in the NOD.IL-$4^{-/-}$ animal despite normal secretory responses implies that the loss of function relies heavily if not solely on the presence and function of autoantibodies.

In vitro studies using the SMG C6 cell line have helped to dissect the signaling events involved in secretory dysfunction. Signal events associated with apoptosis do not appear to play a role in secretory suppression. The treatment of cells with serum IgG or monoclonal antibodies against the muscarinic receptor, a proposed autoantigen in SS, do not activate SAPK. Caspase-3 activity was not detected to any significant levels in response to antibody treatment. However, exposure to anti-muscarinic monoclonal antibodies disrupted the ability of aquaporins to translocate to the plasma membrane in SMG C6 cells. Assuming that anti-muscarinic antibodies play an important role in the reduction of gland function, then it would appear that they accomplish this effect by inhibiting intracellular signaling.

CHAPTER 1
INTRODUCTION

Autoimmunity

Autoimmunity is typically thought of as an individual's immune system experiencing a failure to maintain self-tolerance and mounting a destructive assault on one's own body. Autoimmune diseases can be categorized into systemic and tissue specific disorders. Type I diabetes and Hashimoto's thyroiditis are prime examples of tissue specific autoimmune diseases, responding against tissue specific autoantigens, while scleroderma, rheumatoid arthritis, and systemic lupus erythematosus are classic examples of systemic disorders and are thought to respond to more ubiquitous autoantigens such as DNA, or extracellular matrix (ECM) molecules in the connective tissues. The cause of autoimmunity still remains largely unknown, although there are numerous theories as to the mechanisms and factors involved in the initiation of the immunological attack on "self" tissues. It is known that the occurrence of autoimmunity is a result of the combination of both intrinsic and extrinsic factors.

Genetic studies have sought after predisposing defects in both the immune system as well as the target organs in search of factors contributing to the initiation of autoimmunity. Correlations have been established between disease prevalence and certain major histocompatibility (MHC) haplotypes associated with specific diseases.[1,2,3]

Nonetheless, these MHC and other immuno-genetic associations cannot independently account for disease occurrence. Potential defects in target tissues have also been explored as a driving or contributing force behind autoimmunity. However, autoimmune pathologies typically exhibit a delayed onset, suggesting that they are not due merely to a lack of tolerance within a specific tissue or organ, but that the initiation of the disease is reliant on additional factors or events. Furthermore, rare clinical manifestations where an autoimmune disease develops in one but not the other identical twin emphasize that genetic predispositions cannot be solely responsible for disease occurrence.[4]

Extrinsic or environmental factors are also thought to be important in the onset of many autoimmune diseases. Thus, several popular theories on the development of autoimmunity are founded on the premise that the etiological agent responsible for disease onset is an environmental pathogen. These theories suggest that a typical infection in a predisposed individual will initiate an immune response that additionally develops a persistent autoreactive component. The sequestered antigen hypothesis, for instance, proposes that an infection coincidentally exposes the ensuing immune response to tissue specific antigens that the immune system has not been previously tolerized against. Similarly, the antigenic mimicry theory implies that the collection of antigens against which the immune system responds in the eradication of a pathogen contains a protein that is similar to a host protein, such that an autoreactive crossover response occurs.

The one common thread to all autoimmune diseases is that they still remain a mystery in terms of both cause and cure. With respect to this, the advances in our understanding of any one autoimmune disorder may potentially lead to breakthroughs in other diseases.

Over the last several years, Sjogren's syndrome, an autoimmune disease affecting the exocrine glands, has emerged as a promising model of autoimmunity in that significant progress has been made in dissecting the underlying factors and components that lead to disease onset.

Clinical Features of Sjogren's Syndrome

Sjögren's syndrome (SS) is classically defined as a chronic autoinflammatory disorder primarily affecting the salivary and lachrymal exocrine glands. The result of this autoimmune attack is a destruction of the acinar components leading to a dramatic decrease in secretory capacities of these glands, termed xerostomia and keratoconjunctivitis sicca, or dry mouth and dry eye. While the principle target of the immune system in this disease appears to be these exocrine tissues, numerous other symptoms, referred to as extraglandular manifestations, have been attributed to this disease. These extraglandular manifestations include, but are not limited to, the cardio-vascular, renal, and central nervous systems.

While the loss of exocrine secretory function may not seem extremely debilitating to the patient's quality of life, there are severe implications associated with the secondary complications of this disease. More problematic oral complications include an array of opportunistic infections, such as oral candidiasis (affecting approximately one third of SS patients),[5] and increased periodontal disease due to the lacking antimicrobial and tissue regenerative components of saliva. Similarly, in the ocular environment, patients are more susceptible to opportunistic infections due to the decreased presence of antimicrobial factors such as IgA, IgG and lysozyme. Additionally, surface dryness can lead to corneal erosion and eventual blindness. Perhaps the most severe complication of

SS, occurring in approximately 5% of patients, is the development of non-Hodgkin's lymphomas,[6,7] as a consequence of the lymphoproliferative aspects of SS.

As an autoimmune disorder, SS is categorized as a systemic autoimmune disease along with systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). Like many autoimmune diseases, SS many times manifests in association with another autoimmune disease, typically SLE, RA or immune dependent diabetes mellitus (IDDM). In these cases, it is referred to as secondary SS, as opposed to primary SS, which refers to the independent occurrence of the pathology. Also, as with most autoimmune disorders, SS presents with a gender bias, with women seemingly much more susceptible (9:1 female to male ratio).

Complicating efforts to further understand this disease, SS tends to develop later in life. The mean age of onset tends to fall around 53 years of age, although, with heightened awareness of the disease symptoms among the clinical community, the mean age is dropping. The apparent difficulty in the diagnosis of the disease may relate to the continued disagreement among the medical community as to the establishment of a universal set of diagnostic criteria for the disease. Currently, there are several adopted standards, which include the San Diego,[8] Copenhagen,[9] European Community,[10] Greek[11] and Japanese Criteria.[12] These standards all agree on the inclusion of a measurement of salivary and lachrymal gland hypofunction, considering objective and subjective tests inclusively. Exocrine hypofunction is measured both subjectively, in the form of a questionnaire, as well as by several defined functional exams. Lachrymal dysfunction can be ascertained by either the Schirmer-I Test, measuring tear secretion rates, or by the Rose Bengal Staining test, which measures ocular abrasions as an indicator of surface

dryness. Salivary dysfunction is typically measured in several ways, which include stimulated saliva flow, sialography generating a static picture of the parotid gland, or scintigraphy, which provides a dynamic measure of function of the parotid gland. The more stringent standards also require the presence of a positive serological test identifying abnormally increased titres of autoantibodies such as the antinuclear antibodies Ro (SS-A) and La (SS-B) and a labial gland biopsy showing an abnormal focal score of ≥ 1. Focal scoring of the labial biopsy refers to the enumeration of the lymphocytic foci within a $4mm^2$ section of tissue, where a focal infiltrate is defined as the accumulation of 50 or more lymphocytes in a cluster.[13] The differences in the diagnostic standards lead to a wide variability in the diagnosis of SS patients regionally worldwide and significantly different rates of diagnosis even within a defined population when multiple standards are applied to a specific patient group for comparative purposes. This could conceivably be a source of contradiction in the study of SS through patient populations. For this reason, the results of patient studies must be evaluated with the consideration of which diagnostic criteria were used.

Due mainly to our general lack of understanding of underlying pathogenesis of SS, treatment of the disease has historically met with only limited success. Most approaches in treating SS patients target the relief of oral and ocular dryness. Along these lines, strategies have been suggested or employed involving fluid replacement (with synthetic tear and saliva substitutes, for example), mechanical stimulation such as chewing gum, drug-induced stimulation such as pilocarpine hydrochloride.[14] Additionally, the mucolytic drug bromhexine[15] has been used as a therapy although it does not appear to have any salivary stimulatory properties. Systemic anti-inflammatory drugs, in attempts

to suppress the underlying autoimmune response, have not produced many positive results, especially in terms of functional restoration.[16] However, evidence has recently surfaced suggesting the effectiveness of low doses of IFN-α in treatment and functional restoration of salivary dysfunction in SS.[17] Additionally, treatment regimens have focused on ameliorating some of the secondary complications of SS, including the increased susceptibility to opportunistic infections such as oral candidiasis, which affects approximately one third of SS patients.

Animal Models

Due to the difficulties of diagnosis of SS and the progression of the disease into later stages of pathogenesis by the time patients are diagnosed with the disease, it is virtually impossible to determine the initial and early events of SS pathogenesis leading to the onset of overt disease. Therefore, it has become increasingly important to establish a sound animal model for the purpose of the further elucidation of the primary events in the pathogenesis of SS. Along these lines, several mouse models have been proposed as adequate models of the disease. These mouse models include the MRL/*lpr*,[18] and NFS/*sld*[19] strains, as well as the TGF-β gene-knockout[20] and the graft-versus-host mouse[21] model. All of these mouse models have been employed in the study of SS based primarily on the presence of focal lymphocytic infiltration in the exocrine tissues and the detection of serum autoantibodies. More recently, the NOD mouse has emerged as a more appropriate model of Sjogren's syndrome[22] based on these same criteria, and that it additionally loses exocrine secretory capacity corresponding to immune infiltration of the salivary and lachrymal glands. However, in light of the fact that autoimmune diabetes, which also occurs in the NOD mouse, has been implicated as a cause of secretory

hypofunction through the effects of glucose dysregulation, the NOD.B10.*H-2*^{b} mouse was characterized and presented as the first naturally occurring model of primary SS. In this congenic derivative of the NOD animal, the MHC region containing the I-A^{g7} locus closely associated with autoimmune diabetes has been replaced by the C57BL/10 allele I-A^{b} and as a result, the SS-like pathogenesis remains intact while the diabetes ceases to develop.[23] In the context of these animal models, many aspects of the early events and insights into the underlying pathogenesis have come to light.

One of the more significant realizations to surface from the study of these mouse strains centers around the evidence that SS may not be entirely the result of an aberrant immune response directed at the salivary and lachrymal glands. In the characterization of another congenic derivative of the NOD mouse, the NOD-*scid*, the salivary glands displayed a disrupted morphology, with a depletion of acinar cells and a replacement or hyperproliferation of the ductal structures (as seen also in the salivary tissues of SS patients), despite the absence of lymphocytes or any obvious immune-mediated attack on the exocrine tissues.[24] As further confirmation of the original findings in the NOD mouse relating loss of secretory capacity with the presence of lymphocytes, these animals retained normal salivary function throughout life in the absence of any detectable immunological infiltrate. These observations led to the conclusion that the development of the SS-like pathology in the NOD mouse was dependent on a combination of an underlying developmental or genetic defect within the exocrine glands and an immune infiltration in response to this defect, although this did not rule out the possibility that the SS-like disease in these mice is also dependent on an abnormality in the immune system. As a model for SS, it has yet to be ascertained whether the human disorder is truly a

result of a similar glandular defect, essentially because the specific underlying genetic defect in the NOD mouse has not yet been identified to allow for correlation studies in SS patients. However, just as in the NOD mouse, salivary biopsies of patients with SS provide evidence of apoptotic events in remote locations from lymphocytic infiltrates, and changes in matrix metalloproteinase (MMP) activity and extracellular matrix (ECM) constituent expression are also noted in SS patients.

Etiological Factors

Based on the ongoing studies of both SS patients and the mouse models of the disease, our understanding of both the initiation and development of SS has advanced markedly. As the disease becomes further defined, we become more aware that the development of SS depends on both intrinsic as well as extrinsic factors. Evidence exists to suggest certain genetic links to the onset of SS, although family studies have shown repeatedly that genetics are not the sole indicators and that the immune system relies on additional triggers that apparently are derived from the environment.

Perhaps the most significant genetic linkage associated with SS involves the major histocompatibility (MHC) locus. While specific studies have mapped an increase in the prevalence of certain MHC haplotypes, these documented trends typically have been associated with defined ethnic groups. For instance, SS correlates to increased representation of HLA-DR3 and DQ2 in Caucasians.[1] However, there are variances in the associated MHC haplotypes when looking at other ethnic groups such as Chinese, Japanese and Greek populations, which are affiliated with an elevated representation of HLA-DR8 and HLA-DR5.[25,26] Overall, though, the incidence of SS tends to be associated with an increase in the expression of the HLA-DR alleles.

Non-immunological factors influencing the development of Sjögren's syndrome include hormonal regulation, suggested by the sexual dimorphism (9:1 in favor of females over males) found in the patient population. This has been supported by numerous lachrymal studies in models such as the MRL and NZB/NZW mouse strains where androgens tend to exert a protective influence for dacryoadenitis.[27] Of note, the NOD mouse presents a partial reversal of androgen influence, where the presence of androgens correlates to increased lachrymal destruction.[28,29] In the case of the influence of the sex hormones on disease pathogenesis, it is worth emphasizing that while endocrine regulation may not itself be directly immunological in nature, studies have demonstrated the abilities of estrogens and androgens to influence the immune response[30,31] and this regulatory capacity is certainly important in the pathogenesis of SS. However, if the mouse models are a true reflection of essential events in SS, then hormonal influences may be modulating a predisposed sub-threshold immune response to push it beyond the limit or, conversely, suppressing an immune response to a point just under the threshold of activation that would result in the pathological state.

Additional potential non-immunological factors contributing to the tissue specific attack include the increased MMP activity and elevated levels of apoptosis[32] detected in the exocrine glands, suggestive of an underlying disruption of the ECM.[33,34,35] Furthermore, increases in laminin expression have been detected in SS labial gland biopsies prior to lymphocyte infiltration.[36] Disturbances in the ECM introduced by the aberrant MMP activity destabilize the cells and promote their progression into an apoptotic phase.[37] Conceivably, this could contribute to the recruitment of the immune system to precipitate the autoimmune response.

Environmental factors have been studied in association with events leading to localized immune activation and to immune dysregulation and disease onset. Viral-induced onset of disease tends to receive a significant amount of attention and, along these lines, several viral pathogens have been identified as potential etiologic agents for Sjögren's syndrome. Several members of the gamma herpesvirus family, most notably Epstein-Barr virus (EBV)[38,39] and human herpesvirus type 6 (HHV-6),[40] have been detected at a slightly higher frequency in Sjögren's syndrome patients. Sicca symptoms have also been associated with elevated serum antibody titres against hepatitis C virus (HCV).[41] Despite the extensive studies on viral etiologic agents leading to Sjögren's syndrome, no single virus has been conclusively identified as a direct causative agent, and quite possibly, multiple chronic insults may act in concert to trigger the necessary immune activation leading to autoimmune exocrinopathy.

Immunopathology of SS

The immune response involved in the attack on the exocrine tissues in SS patients highlights a number of consistent, key features of autoimmunity, in general. The lymphocytic infiltrates in the salivary glands have been characterized, based predominantly on labial gland biopsies, and show a consistent composition between patients. Serological examinations have demonstrated several commonly occurring autoantibodies in the pathogenesis of the disease. To this extent, both histology and serology have been included as parameters in several standard sets of criteria for the diagnosis of SS.

Histological evaluation of the exocrine glands reveals several distinct anomalies associated with the onset of disease. Certainly, the loss of function has been correlated to

the development of focal lymphocytic lesions in the glands[42,43] and these foci form as peri-ductal infiltrates. However, additional studies repeatedly have been unable to show a significant correlation between severity of infiltration and loss of gland function.[44] In addition to the accumulation of lymphocytes, the gland appears to lose as much as 50% of the acinar cell population and these cells are replaced by ductal structures.[45] One possible explanation for this morphological rearrangement is that the ductal epithelial cells have been proposed to be the progenitor cells for the acinar population[46], thus suggesting that the hyperproliferation of the ductal cells may be an attempt to replenish the dying acinar structures within the gland. Immunohistochemical analysis of the glandular epithelial cells indicates that these cells are apparently active participants in the immune response, expressing HLA-DR,[47] B7.1 and B7.2[48] and thus potentially serving as an additional antigen-presenting cell to drive the T cell activation. Thus, not only are the infiltrating lymphocytes contributing to the suppression of secretory function, but the tissue epithelial cells appear to be playing an active role in their own demise and subsequently contributing to the loss of secretory capacity.

Immune Cell Composition of Focal Infiltrates

The focal infiltration of the salivary glands has been much more extensively studied than that of the lachrymal glands, probably due to the relative accessibility of the minor salivary glands and non-invasiveness of the biopsy procedure. However, characteristics of the focal infiltrates have been supported by evidence from studies in the various mouse models, which demonstrate similar proportions of immune cells. In the human disease, lymphocytes comprise approximately 80% of the focal lesion[49] and are predominantly $CD4^+$ $\alpha\beta$ T cells. Plasma B cells present in the lesions produce mostly IgM and IgG[50]

and have a greater than normal potential to mutate into non-Hodgkin's lymphomas.[51] Similar to the human condition, the salivary infiltrates in the NOD mouse are comprised of approximately 45% T_H cells, 15-20% B cells, and 10-15% $CD8^+$ T cells, while in the lachrymal glands, the populations are skewed slightly in favor of the B cell (23-30%), with the T_H cells constituting another 25-30%.[52] The T cell populations appear to demonstrate restricted V_β usage, with an increase in $V_\beta 6$ and $V_\beta 8$.[51] In addition to the lymphocytes, the infiltrates also contain small but significant macrophage and dendritic cell populations. In the NOD mouse, but not the MRL/*lpr* model of SS, the development of autoimmune lesions is preceded by an influx of $CD11c^+$ dendritic cells.[53]

Cytokine Expression

Cytokine expression in the salivary glands of Sjögren's syndrome patients as well as that observed in various mouse models suggests a pro-inflammatory profile. Cytokine mapping in the salivary and lachrymal glands has relied largely on the measurement of stable mRNA levels via reverse transcriptase polymerase chain reaction. Evaluation of patient populations reveal the expression of IL-1β, IL-2, IL-6, IL-10, IL-12, IL-18, TNF-α, TGF-β, and IFN-γ in the minor labial glands.[54,55] Similarly, studies in the mouse models report the expression of IL-1β, IL-2, IL-6, IL-7, IL-10, IL-12, TNF-α, TGF-β, iNOS and IFN-γ in the submandibular and lachrymal glands.[51,56,57] In addition, IL-5 is noted in the murine lachrymal gland. Rarely, IL-4 can be detected but is generally considered absent in the infiltrated glands. More recent studies in both human and mouse salivary glands have indicated that this comprehensive pro-inflammatory pattern of cytokine expression can be detected in the salivary tissues of healthy donors as well as non-autoimmune mouse strains at levels similar to disease state values.[58,59] It would

appear, therefore, that rather than being a defining hallmark of the disease process, these cytokine mRNA expression profiles are more a reflection of tissue specific micro-regulation of the immune responses favoring a pro-inflammatory response in these glands in the context of localized immune activation. In support of this, Fox *et al.* (1998) showed that acinar and ductal epithelial cells express a wide variety of cytokine mRNA including IL-2, IL-6, IL-10, TNF-α, IFN-γ, and TGF-β[60] while other reports indicate that IFN-γ can induce upregulation of IL-1β, IL-6 and TNF-α, along with HLA-DR and ICAM-1 in HSG cells.[61,62] This also supports the theory that the glandular epithelial cells are not only the target of the autoimmune attack, but they also play an instructive role in the disease. In contrast to mRNA levels, the protein expression of the infiltrating lymphocytes and epithelial cells in murine salivary glands, as measured by immunocytochemistry, is markedly different between NOD versus Balb/c control strains. In the case of the pro-inflammatory cytokines, significantly greater quantities can be detected in association with the Sjögren's syndrome-like disease state.[27] In essence, this accentuates an extremely important point, i.e., while mRNA levels in a cell may reflect gene activation, gene expression and gene product activity are regulated on many levels beyond transcription by cellular manipulation of translation rates, post-translational modifications, and even product sequestration. Therefore, mRNA expression levels may not necessarily equate with protein expression. In fact, this appears to be the case in terms of glandular cytokine expression. Regardless, the combination of mRNA and protein expression data support the conclusion that SS is associated with an elevation in the expression proinflammatory cytokines in the target glands.

The Humoral Response

Sjögren's syndrome patients also present with elevated levels of antibodies, or hypergamma-globulinemia, as a result of the lymphoproliferative nature of the disease. The first SS-related autoantibodies identified were those against the anti-nuclear antigen SS-A/Ro and SS-B/La.[63] Depending on the stringency of testing, these autoantibodies can be detected in up to 90% of SS patients,[64] hence their general inclusion in the diagnostic criteria for SS. The slight inconsistency in the presence of the autoantibodies may be a reflection of the waxing and waning nature of this chronic autoinflammatory disease. Additionally, patients diagnosed with SS will many times test positive for rheumatoid factor.[65] In the last 10 years, a vast assortment of autoantigens have been identified encompassing intracellular, cell surface, and secreted proteins. This growing list of autoantigens includes parotid secretory protein (PSP),[66] carbonic anhydrase II,[67] α–fodrin,[68] and the muscarinic[69,70] neuroreceptors. As of yet, none of these agents has definitively been associated with loss of secretory function or any other aspects of the progressive rheumatological pathology.

Apoptosis

Over the last several years, aberrant apoptotic activity has been suggested as playing a supportive role in autoimmunity. In the case of Hashimoto's thyroiditis, the abnormal expression of Fas on thyrocytes, cells that constitutively express FasL, leads to a malfunction in immune privilege established in the thyroid gland resulting in fratricide that draws the attention of the immune system[71] and recent studies have shown similar trends in thyrocytes from Grave's disease patients.[72] And many other autoimmune diseases, including rheumatoid arthritis[73], autoimmune diabetes,[74] and systemic lupus

erythematosus,[75] depend, in some way or another, on the disregulation of normal apoptotic activity. SS, like so many other autoimmune diseases, also appears to involve aberrant apoptotic activity.[76]

Apoptosis is a natural process in multicellular organisms whereby the old or excess cells undergo a controlled, programmed cell death (PCD). Unlike necrosis, cellular apoptosis seems to be a more methodical self-destruction, which reduces the inflammatory repercussions with regards to the immune system in the immune-mediated clearance of apoptotic bodies. Apoptosis has been intensely studied and has consequently been associated with some distinct biochemical and morphological features. During the course of PCD, cells undergo nuclear condensation and breakdown with the digestion of chromosomal DNA,[77] while the plasma membrane loses surface integrity and forms blebs.[78] Like the nuclear and plasma membranes, the mitochondrial membrane loses its integrity and membrane, thus contributing to the disruption of the cell by further disturbing the ion balances within the cell cytosol.[79] Eventually, the cell fragments into smaller apoptotic bodies, membrane-encapsulated remnants of the dead cell, which are taken up by circulating macrophage or neighboring cells.[80,81,82]

These morphological changes in the dying cell are occurring synchronously with a number of important biochemical activities, many of which are the causative agents of the morphological appearance. The cell will be induced to undergo apoptosis in response to any one of numerous cell signals, from the disruption of attachment to the extracellular matrix,[33] to the deprivation of growth factors, to the specific induction by the activity of certain receptors such Fas and TNF-α[83] or the response to various cytokines.[84,85] Regardless of the source, the signal cascade tends to follow a general path that involves

several relatively conserved checkpoints and ultimately results in the activation of a specific family of proteases called caspases.[86] Caspase activation, and more specifically caspase-3 activation, tends to be considered a point-of-no-return in the commitment to PCD.[87]

Caspases are a family of cysteine proteases consisting of approximately 14 known members,[77] which have been associated with the various pathways that ultimately result in PCD. This family has been broken down further, most member being sorted into one of three sub-categories – cytokine activators, initiators, and effectors (see Figure 1-1). Caspase-1, also referred to as ICE or Interleukin-1β Converting Enzyme, was the first identified and was classified based on its ability to cleave the proactive form of IL-1β to yield an active cytokine.[88] Other caspases of interest include caspase-8, linked to Fas/FasL death signaling, caspase-9, which is activated by cytochrome-c released from the mitochondria, and caspase-3, an important effector caspase which is activated in virtually all cells undergoing PCD and is known to be responsible for the activation of mechanisms that lead to membrane blebbing[70] and DNA degradation.[68] Another important family of proteins in the regulation of apoptotic activity in a cell is the bcl-2 family. There are at least 20 proteins that have been classified in this family based on strong sequence homology.[89] The bcl-2 family can be further divided into pro-apoptotic and anti-apoptotic members based on their function. The two most well studied members of this family are bcl-2, a protective member, and bax, a pro-apoptotic member, and additionally, bcl-X_L and bad have received a lot of attention. Many of the bcl-2 proteins can be found on the mitochondrial membrane surface where they interact with each other.

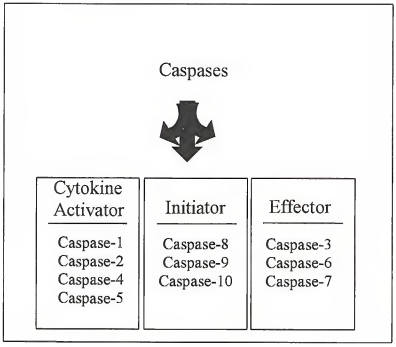


Figure 1-1. Categorization of the caspase family of proteases.

The mechanism by which they determine a cell's progression into PCD relies on a dynamic equilibrium established between pro-apoptotic and anti-apoptotic members. Original paradigms depicted a simple ratio between bax and bcl-2 as a way in which the cell decided whether to progress into PCD; however, this system is not nearly so simple, as the activity and interactions of these proteins can be modified not only by other members of the family but also by phosphorylation events.

Of all the death signaling pathways, the Fas-mediated pathway has been the best characterized. Fas is a cell-surface receptor which, when interacting with Fas ligand (FasL), initiates a signal cascade that ultimately kills the cell.[76] Through the interaction with FADD via these proteins death domains (a conserved amino acid sequence motif), Fas sends a signal that activates caspase-8, an initiator caspase that cleaves and activates caspase-3.[90] The Fas pathway has been implicated as an important factor in such processes as activation-induced cell death of lymphocytes in immune regulation[91] and also in the cytotoxic T cell killing mechanism[92] in the case of the cell mediated immune response. As this would suggest, most cell types throughout the body express or can be induced to express Fas.[93]

In the context of the mouse models of SS, and especially the NOD mouse, elevated levels of apoptosis have been detected in the exocrine tissues early on in the disease pathogenesis.[32] Increases in cysteine protease activity can be detected in the saliva of pre-diabetic and diabetic NOD mice, indicating enhanced levels of apoptosis within the salivary glands of these animals. Further histological analysis of the salivary and lachrymal glands reveals the upregulation of Fas on the epithelial cell surface in the presence of constitutively expressed FasL.[94] This agrees with the studies on the human

condition that reveal similar increases in Fas and FasL expression in these tissues.[95] While abnormal Fas expression may begin to account for the disappearance of the acinar cell population in the course of the disease, elevated levels of bcl-2 in the invading lymphocytes and ductal epithelium may help to account for the persistence of these cells in the lesions.[96,97,98] Finally, in the NOD and NOD-*scid* mouse strains, immunohistochemical analysis of the salivary ductal and acinar cells reveals an increased presence of the pro-apoptotic bax protein and activated caspase-3.[99] This evidence together strongly suggests an important role for apoptosis in the pathogenesis of autoimmune exocrinopathy, and the fact that much of this activity has been observed in the NOD-*scid* background implies that the increases in apoptotic activity are a result of a glandular defect which may be driving the autoimmune processes, rather than strictly of a immunological attack on the glands.

Autoantibodies and Apoptosis

While the presence of apoptotic activity in the salivary and lachrymal glands has been well established in the pathogenesis of SS, the exact causes still remain undetermined. The observations in the NOD-*scid* mouse certainly suggest that there is an underlying genetic or developmental defect in these tissues that will lead to increased PCD within the glands. However, the immune infiltrates could also potentially be contributing to the increases in PCD and exacerbating the situation. Through the studies in the NOD mouse, a timeline of disease progression has been developed detailing many critical events associated with the deterioration of the glands and the eventual loss of function. On this timeline, the elevation in caspase activity and the initial appearance of lymphocytic foci in the glands occur concomitantly around 12 weeks of age. This lead to an extremely

important question: is the immune infiltrate contributing to the destruction of the gland and functional suppression by further accelerating epithelial apoptosis.

Additionally, as evidenced in the NOD-*scid*, the lymphocytes are somehow responsible for mediating the loss of exocrine function. Theoretically, this could occur by several different means – cytotoxic T cell (CTL) activity directly killing the secretory acinar cells, secreted cytokines killing or otherwise suppressing these cells, or by the activity of specific autoantibodies interacting with the acinar cells. Considering that salivary gland hypofunction is usually disproportionately greater than tissue infiltration, and additionally, that the parotid gland of the NOD mouse appears histologically normal despite the dramatic decrease in salivary output in these animals, CTL-mediated functional suppression seems unlikely. Both cytokines and antibodies represent soluble factors that could surpass the suppression of only the secretory cells localized near lymphocytic infiltrates. Along this line, much attention has been given to cytokine-mediated suppression, due to the glandular cytokine profiles and inflammatory nature of SS. To date, there is little evidence for a functional relevance for the anti-nuclear autoantibodies (the most well-characterized of the SS-related autoantibodies), and direct functional studies have not been performed using any other reported SS autoantibodies. But in consideration of the new autoantigens and autoantibodies that have more recently been associated with SS, this may certainly be a faulty conclusion and, hence, the issue deserves more attention. This concern becomes more relevant upon realization that several of the autoantibodies identified in the last 6 years appear to recognize the muscarinic and β-adrenergic neuroreceptors, centrally responsible for the fluid and proteinaceous phases of saliva and tears, respectively.

Therefore, the scope of this study is to approach the question of the relevance of the autoantibodies in SS and to assess their ability to drive loss of function. More specifically, the NOD mouse and congenic partner strains will be used as an *in vivo* model system to assess the contribution of the autoantibodies to the loss of function and to determine if the mechanism by which exocrine function is suppressed is dependent on the induction of PCD in the acinar cells in an immune-dependent or antibody-dependent fashion. The specific directives of this research will be to explore the following topics:

1. Evaluate the role of the autoantibodies in the loss of glandular function in SS.
2. Further define the regulation of the humoral arm of adaptive immunity on the generation and function of the autoantibodies as well as the development of autoimmune exocrinopathy in the NOD mouse through the use of cytokine-deficient congenic strains.
3. Evaluate the role of apoptosis in the onset of secretory dysfunction in the NOD mouse model of SS.
4. Define whether SS autoantibodies play a role in the observed increase in apoptosis in the exocrine glands.

CHAPTER 2
IMPORTANCE OF B CELLS IN SJÖGREN'S SYNDROME

Introduction

Sjögren's syndrome is an autoimmune rheumatic disease primarily targeting the salivary and lachrymal glands.[100,101] It is often a complication of connective tissue diseases such as rheumatoid arthritis or systemic lupus erythematosus. Although the histopathology of the tissue is characterized by the presence of lymphocytic infiltrates consisting of CD4+ T cells, a minor component of CD8+ T cells, B cells, and macrophages, the patho-physiological outcome is the loss of exocrine gland secretory function.[99,100] Patients with Sjögren's syndrome additionally demonstrate hypergammaglobulinemia on serological analyses, with a range of autoantibodies reactive against cell surface, cytoplasmic and nuclear proteins of exocrine tissue.[68,100,102] Although the pathogenesis of autoimmune sialoadenitis and dacryoadenitis remain unclear, animal models for the histopathology have demonstrated the dependence of this aspect of disease on transfer of activated T cells.[103,104,105]

Despite the identification of numerous mouse models mimicking the histopathology of Sjögren's syndrome, only the nonobese diabetic (NOD) mouse has been found to develop the corresponding clinical outcome of loss of secretory function.[22,106,107] The NOD mouse,

first identified as a model for type I, insulin-dependent diabetes, develops a Sjögren's syndrome-like immunopathology of the exocrine glands.[108] Genetic analyses and immune cell transfer studies have shown that these two autoimmune diseases arise independently in the same animal.[55,103,104,105,109] Cell transfer studies[103,104,105] have demonstrated a major role for CD4+ T cells in the pathogenesis of both these autoimmune diseases, but more recent results have implicated the need for immunologically active B cells in the initiation of the autoimmune process. Noorchashm *et al.*[110] have shown that continuous injection of anti-Igμ antibodies into NOD mice results in the absence of insulitis, diabetes, and sialoadenitis. Additionally, congenic NOD B cell knockout mice, NOD.Igμ^{null}, do not develop insulitis or diabetes, demonstrating a requirement for B lymphocytes in the initiation of the autoimmune tissue targeting of the pancreas.[111,112]

An analysis of the underlying signal transduction response in the exocrine tissues from NOD mice has revealed the specific down-regulation of intracellular second-messenger signaling components adenylate cyclase and phospholipase C.[69,113] This was accompanied by a corresponding reduction in the density of cell surface muscarinic and adrenergic receptors. Further evaluation of sera collected from older NOD mice detected the presence of a population of autoantibodies capable of reacting with the receptors responsible for neural stimulatory initiation of exocrine secretion. A similar set of neurostimulatory autoantibodies has been reported to be present in the IgG pool isolated from primary Sjögren's syndrome patients.[71] Using the congenic NOD.Igμ^{null} mouse, we have investigated the role of B lymphocytes in the pathogenesis of autoimmune exocrinopathy in the NOD mouse model for Sjögren's syndrome. The observations presented here suggest that development of exocrine-gland secretory dysfunction depends

on the presence of B lymphocytes and that autoantibodies generated from these cells are important in the clinical manifestation of xerostomia and keratoconjunctivitis sicca. Furthermore, the appearance of T cells in exocrine tissue, despite the lack of a corresponding insulitis, supports the concept that NOD mice develop two, independent autoimmune diseases.

Materials and Methods

Materials. C57BL/6-scid and NOD/Lt mice (n = four to six animals/group) were bred and maintained under specific pathogen-free conditions in the mouse facility at the University of Florida, Gainesville, FL, and the Forsyth Dental Center, Boston, MA. NOD.Igμ^{null} mice were obtained from David Serreze, The Jackson Laboratory, and bred in the above animal facility. Both male and female mice at 8-12 weeks and >20 weeks of age were used.

Histological evaluation. Parotid, submandibular, and lachrymal glands as well as pancreas were identified by gross morphology and freed of connective tissue and lymph nodes before a 4-hr fixation in 10% formalin and embedding in paraffin blocks. Five-micron thin sections were cut, stained with hematoxylin/eosin, and examined for focal lymphocytic infiltrates by light microscopy.[22,106]

Saliva collection and preparation of gland lysates. Whole saliva was collected after stimulation of secretion by using isoproterenol (0.20 mg/100 g body weight) and pilocarpine (0.05 mg/100 g body weight) dissolved in saline as described previously.[22,106] Saliva samples were collected for 10 min from groups of n = 4 to 6 mice on two separate occasions and then frozen at 80°C. Excised parotid and submandibular glands were homogenized in 10 mM Tris buffer (pH 7.4) and immediately frozen at 80°C. Protein

assays of both saliva and gland lysates were performed using the Bio-Rad protein assay reagent with BSA as the standard.[24] Amylase assays were performed as described previously by using starch as the substrate.[24,106]

Cysteine protease activity. Protease activity in saliva and gland lysates was determined by using a standard protease assay as described elsewhere.[66] The reaction mixture consisted of 25 µl 100 mM sodium benzoyl-dl-arginine-p-nitroanilide (BAPNA) in DMSO, 10 µl unknown sample, 190 µl phenylmethylsulfonyl fluoride (PMSF) buffer consisting of 0.2 mg/ml DTT, 0.5 mg/ml Na_2EDTA, and 1.0 mM PMSF in 100 mM phosphate buffer (pH 6.0). Experimental samples, as well as a dilution profile of papain, were incubated at 37°C for 1 hr. The reactions were terminated by the addition of 25 µl of glacial acetic acid and adjusted to 1.0 ml with ddH2O, and the optical density was determined at OD_{405} to determine the amount of p-nitroaniline released. Each assay was performed in duplicate on three separate occasions.

PAGE and Western blot analysis. Total salivary proteins (15 µg/well) or gland lysates (50 µg/well) were separated on 10% or 12% SDS-polyacrylamide gels.[66,69] Individual submandibular gland lysates from groups of 4- to 8-week-old and 20-week-old mice were prepared by Dounce homogenization in 10 mM Tris buffer (pH 7.5) after removal of lymph nodes and connective tissue. The proteins were transferred to Immobilon-P membranes (Millipore), blocked in TBS containing 3% nonfat milk and 3% BSA, and incubated in a 1:10,000 dilution of rabbit polyclonal antibody to parotid secretory protein, washed three times, incubated with a goat anti-rabbit alkaline phosphatase-conjugated secondary antibody, and reacted with chromogenic substrate as

described previously.[24] All gels were run on two separate occasions for reproducibility of results from individual mice.

IgG preparation and injection into mice. Sera were isolated from whole blood collected from patients diagnosed with primary Sjögren's syndrome (SS) by the loss of saliva and tear production, positive Rose Bengal staining, lymphocytic foci in minor labial gland biopsies, and the presence of antinuclear SS-A/Ro and SS-B/La autoantibodies in serological evaluations.[100,101] At the time of serum collection, the patients were determined to be free of other complicating autoimmune diseases. Age- and sex-matched healthy individuals were used for control sera. The ages ranged from 30 to 68 years of age. Sera were also collected from >20-week-old NOD/Lt mice.

Murine and human IgG fractions were isolated by standard protein A-agarose and DEAE-chromatography, respectively, dialyzed to remove excess salts, and lyophilized to concentrate. The purity of the IgG fractions was assessed at >90% by SDS/PAGE. Normal control mouse IgG was purchased from Sigma and was of >90% purity (ultrapure grade). The final IgG fraction was filter-sterilized and injected into the intraperitoneal cavity of 10- to 12-week-old NOD.Igμ^{null} mice at 100 μg/100 μl PBS.

Preparation of F(ab')$_2$ from human IgG. Antibody fractions isolated by DEAE chromatography were digested overnight with pepsin (1:100 of IgG) in a water bath at 37°C. The preparations were passed over protein-G Sepharose columns to remove undigested antibodies and Fc fragments. The unbound fraction was tested with anti-F(ab')$_2$-specific and anti-Fc-specific peroxidase-conjugated antibodies (Jackson ImmunoResearch) to confirm the purity of the preparation. F(ab')$_2$ fragments were injected into the intraperitoneal cavity of mice at 10 μg/100 μl.

Muscarinic receptor binding assay. Mouse parotid gland membranes were isolated as described previously.[103] Total membrane protein (250 μg) was incubated in a 1.0-ml volume of 50 mM Tris buffer (pH 7.4) containing 10 mM $MgCl_2$ and protease inhibitors[105] at 4°C for 15 hr with no or varying concentrations of human IgG. After the incubation with gentle mixing, the samples were incubated at 25°C for 1 hr with the addition of 5.0 nM [^{3}H]QNB (quinuclidinyl benzilate; Amersham; 5 × 105 cpm/reaction). The radiolabel bound to membranes was collected by centrifugation at 15,000 × g and washed in the above buffer three times before the addition of scintillation mixture and analysis of the radiolabel bound to the membranes by liquid scintillation counting in a Beckman LSC 3801 Counter. Alternatively, membrane receptors were first radiolabeled by incubation with [^{3}H]QNB in the above buffer containing 1.0% Triton X-100 for 1 hr at 37°C, followed by immunoprecipitation with human control or Sjögren's syndrome patient IgG. Receptor-IgG complexes were collected by the addition of Protein A-agarose (Sigma) beads followed by centrifugation as above.

Statistical analysis. All measures of variance are given as SEM by the Shapiro and Wilks test[23] the distributions for saliva and tear volumes as well as protein concentration were found to be normal ($P > 0.05$) and were analyzed by a parametric ANOVA.[24,66] Tests of ANOVA between independent means were not normal ($P < 0.05$) for cysteine protease activity and were performed subsequently with a nonparametric single-factor test by using a SAS computer software program, results in which $P < 0.05$ was considered significant.

Results

Histological Evaluation of NOD.Igμnull Exocrine Glands. Flow cytometric analyses of splenic lymphocytes prepared from NOD.Igμ^{null} mice failed to detect the presence of B cells. At the same time, sera isolated from these mice lacked detectable IgG. These findings indicate that NOD.Igμ^{null} mice used in these studies preserve the knockout genotype/phenotype. Despite the absence of pancreatic autoimmunity in 8-week-old NOD.Igμ^{null} mice, histological evaluation revealed the presence of small lymphocytic infiltrates of both the lachrymal and submandibular salivary glands (Fig. 2-1). Older NOD.Igμ^{null} mice (>20 weeks) revealed the continued presence of focal infiltrates of the exocrine tissues with only an occasional peri-islet or islet infiltration observed. However, none of these mice developed elevated blood glucose levels or diabetes.

Detection of Biochemical Alterations in the Saliva and Exocrine Tissue Lysates of NOD.Igμ^{null} Mice. Hallmark biochemical changes in saliva volume and composition, as well as glandular protein synthesis that occur independently of autoimmunity,[24,66] were observed with increased age of the mice. Secretory function of NOD.Igμ^{null} mice >20 weeks of age showed that stimulated flow rates were not different from those of 8-week-old mice ($P > 0.05$; Table 1), with values similar to those reported previously for healthy control BALB/c and C57BL/6 mice.[22,106] Cysteine protease activity, a marker for acinar and ductal epithelial cell apoptosis, was increased 1.4-fold in the saliva of NOD.Igμ^{null} at 20 weeks of age as compared with mice 8 weeks of age (Table 2-1; $P < 0.01$). Both young and older mice had overall elevated cysteine protease activity in submandibular gland lysates, activities similar to the parental NOD strain. However, further analysis of 8-week-old male mice consistently produced higher levels of enzyme activity than 20-week-

old male or female NOD.Igμ^{null} mice, while 8-week-old female mice had levels of activity similar to that reported previously for BALB/c and young NOD-scid mice.[24,66] Amylase activity, an enzyme produced by the parotid gland acinar cells, was reduced by 32% in older mice ($P < 0.05$), again consistent with the previous observations in NOD and NOD-scid mice.[22,69,106,113] Total protein content of saliva remained constant between the two age groups. Parotid secretory protein, a normal constituent of differentiated parotid gland synthesis, was observed to be aberrantly synthesized and processed in the submandibular gland of NOD.Igμ^{null} mice by 20 weeks of age (4/4), while gland lysates of younger 8-week-old mice either did not produce the protein (2/4) or produced a small amount (2/4) of the normal 25-kDa isoform (Fig. 2-2). Thus, except for the loss of secretory function, development of biochemical and physiological changes consistent with previously described nonimmune components of the exocrine tissue epithelial cell pathology occurs independently of the absence of B lymphocytes in NOD.Igμ^{null} mice.

Transfer of Secretory Gland Dysfunction with Serum IgG Fractions. Both primary Sjögren's syndrome patients and NOD mice produce autoantibodies that interact with the autonomic nervous system receptors responsible for initiating the secretory response.[69,71,113] To determine the potential for IgG antibodies to transfer exocrine tissue dysfunction to young NOD.Igμ^{null} mice, a series of transfer experiments was performed in which IgG from healthy control mice and healthy humans, prediabetic NOD mice, as well as human Sjögren's syndrome patients were infused into NOD.Igμ^{null} mice. As indicated in Fig. 2-3A and B, mice given a single infusion of IgG from nondisease mice or healthy human sera retained near-normal secretory function at 24 hr ($P > 0.05$; $n = 4$) after

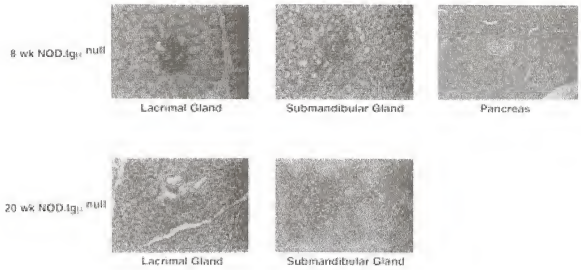


Fig. 2-1. Histological analysis of the exocrine tissues and pancreas from NOD.Igμ^{null} mice. Glands from n = 4 animals per age group were evaluated by two individuals for independent analysis. (200X) .

Table 2-1. Analysis of age-related physiological and biochemical changes in NOD.Igμ^{null} mice.

Animal	Saliva volume	Total protein	Amylase activity	Cysteine protease activity
8-wk NOD.Iμ^{null} (n = 4)	297.6 ± 28.1	13.77 ± 1.98	2,961 ± 256	31.5 ± 4.8/39.7 ± 5.1
20-week NOD.Igμ^{null} (n = 6)	324.8 ± 17.3	13.04 ± 2.57	2,331 ± 241	44.7 ± 3.6/38.8 ± 2.9
20-week NOD*	106.2 ± 10.5	21.6 ± 3.3	1,732 ± 356	45.8 ± 5.3/40.6 ± 4.7

All values expressed as mean ± SE based on two experimental determinations. Volume and protein concentration, collected over a 10-min period, after stimulation of secretion with a cocktail of autonomic agonists isoproterenol and pilocarpine, is expressed as μl/10 min or mg/ml per 100 g body weight, respectively (8, 9). Amylase activity is expressed as mg starch hydrolyzed/mg protein per 100g body weight. Cysteine protease activity is expressed as μg protease/min per mg saliva protein and μg protease activity/min per mg submandibular gland lysate. The significance of differences between mean values was tested by use of a paired ANOVA and Student's t test.

- Values derived from previous studies of the parental pre-diabetic NOD mouse (8, 9, 17, 18).

secretory stimulation with a secretagogue mixture of β-adrenergic and muscarinic/cholinergic receptor agonists.[106] Mice treated with IgG isolated from older, parental NOD/Lt mice secreted only 30% of the saliva volume normally generated. Treatment with IgG from four different Sjögren's syndrome sera (#77, #36, #93, and #45) resulted in an average 54% decrease in saliva volumes generated, relative to the control, after 24 hr (Fig. 2-3B; $P < 0.001$). A 37% decline was noted when purified F(ab')2 fragments from autoimmune patients were given to mice at a concentration of 10 μg/animal. Injection of the Sjögren's syndrome sera IgG into an unrelated mouse strain, C57BL/6-scid, again resulted in a decrease in secretory response after challenge with secretory agonists (Fig. 2-3B). Repeated infusion of normal human IgG caused a decrease in volume collected with time in the control animals (Fig. 2-3C, 20%; $P < 0.05$), which was most likely a result of the stress of constant handling of the animals for injection and the saliva collection regimen. More interesting was the observation that 1 week of continuous treatment of mice with individual Sjögren's syndrome serum IgG showed a sustained loss of secretory function between 40 and 60% ($P < 0.001$). Whereas 7/10 patients' sera caused a significant loss of function 24 hr after the first injection of IgG, 1/10 patient's IgG fraction was capable of stimulating an increase (60-100%; $P < 0.001$) above initial saliva volumes after 24 hr, and another two patients' IgG fraction stimulated saliva secretion in all subsequent injections (Fig. 2-3C). The recovery of saliva production in some of the mice with continuous infusion of IgG is not understood at this time. An additional 1-week washout period with injection of normal human IgG every 48 hr

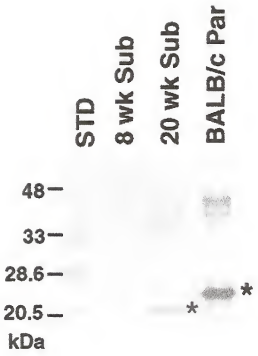


Fig. 2-2. Western blot detection of parotid secretory protein in gland lysates prepared from 8- and 20-week-old NOD.Igμ^{null} mice. Fifty micrograms of tissue lysates was separated by SDS-polyacrylamide gels. The normal 25-kDa BALB/c isoform and the NOD-specific 20.5-kDa isoform are indicated by asterisks. Sub, submandibular gland; Par, parotid gland.

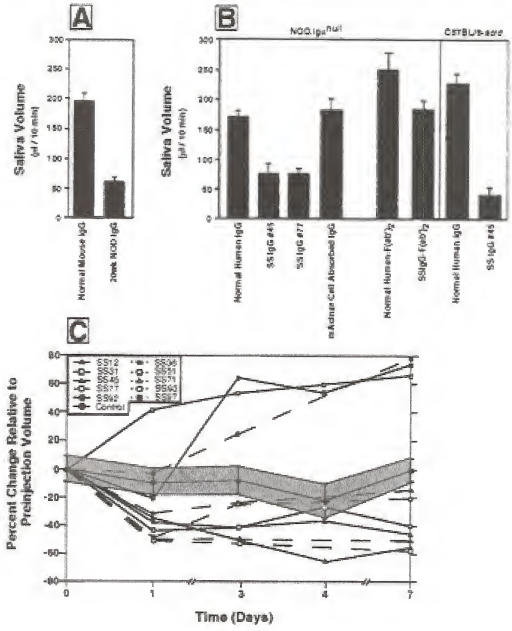


Fig. 2-3. Alteration in the levels of saliva volume generated in NOD.Igμ^{null} mice after a single or repeated injection of normal serum IgG, parental NOD, or Sjögren's syndrome serum IgG fractions. (A) Histogram of saliva volume collected 24 hr after injection of NOD.Igμ^{null} mice with normal or NOD/Lt sera IgG fractions. (B) Histogram of saliva volume after injection with four normal human or four patient IgG fractions. Salivary flow was collected from the oral cavity beginning 1 min after stimulation (n = 4 mice/injection regimen). Mice were injected with 100 µg IgG or 10 µg F(ab')$_2$. All values are expressed as injected for 1 week with normal human IgG (n = 5) or individual Sjögren's syndrome patient (n = 10) IgG fractions.

demonstrated that the volumes of stimulated saliva secretion returned to normal levels in mice treated with Sjögren's syndrome sera and, in some cases, resulted in an overcompensation of volume generated after agonist injection. As an additional control, Sjögren's syndrome patient IgG fractions were preadsorbed against intact murine salivary gland cells before injection into mice. This resulted in the loss of the ability to induce secretory dysfunction (Fig. 2-3B).

Histological Changes in Acinar Cells After Repeated Antibody Treatment. Examination of exocrine tissues from mice after chronic treatment with IgG fractions of Sjögren's syndrome patient sera revealed the presence of vacuolar structures predominantly in the acinar cell portions of lachrymal, submandibular, and, to a lesser extent, pancreatic tissue (Fig. 2-4). Observation at higher magnification (400x) clearly showed that vacuolated cells contained condensed nuclei and vacuolar cell structures with indistinguishable cytoplasmic contents surrounded by membranes. Furthermore, there were greater numbers of nuclei whose appearance suggested apoptosis, consistent with previous biochemical studies.[41,66] These vacuolated cells were not detected in significant numbers in mice repeatedly infused with normal human IgG, nor in the ductal cells or pancreatic islets of the Sjögren's syndrome IgG-treated mice (Fig. 2-4). Similar structural changes in exocrine tissue histology was observed after the injection of $F(ab)_2$.

Muscarinic Receptor Interaction with Sjögren's Syndrome Patient IgG Fractions. Direct interaction of the antibody fraction with salivary gland muscarinic receptors was demonstrated by dose-dependent competition for receptor binding by using radiolabeled agonist. As indicated in Fig. 2-5, incubation of IgG fractions from Sjögren's syndrome patients, but not from healthy controls, inhibited the subsequent binding of the

muscarinic receptor agonist, [^{3}H]QNB, to mouse parotid gland membrane preparations in a dose-dependent fashion. When muscarinic/cholinergic receptors first were incubated with radiolabeled agonist, solubilized with nonionic detergent, and immunoprecipitated with antibody fractions, prior binding of agonist prevented subsequent immunoprecipitation with Sjögren's syndrome patient-derived reagents (Fig. 2-5). This is consistent with the interpretation that both the muscarinic receptor ligand and a population of patients' sera IgG compete for interaction with the receptor agonist-binding site.

Discussion

A requirement for B lymphocytes in the pathogenesis of Sjögren's syndrome-like disease is suggested by the fact that NOD.Igμ^{null} mice retain full secretory capacity on stimulation with autonomic receptor agonists, similar to previous observations with the congenic, immunodeficient NOD-scid mouse. The role of secreted autoantibodies rather than B cells as antigen-presenting cell phenotype appears to be potentially more important in the ability of NOD and Sjögren's syndrome patients to develop the loss of secretory function. The use of isolated IgG fractions to produce salivary gland dysfunction in immunodeficient mice suggests that this component of the immune system is a critical mediator for clinical presentation of sicca complex. Autoimmune exocrinopathy often is thought to result from cytotoxic effects of activated T lymphocytes or cytokines in response to programmed cell death occurring in the glandular epithelial cells.[95,114,115,116] However, the present results indicate that although T cells may be involved in the pathology of the exocrine tissue in NOD mice, the B cell effector arm appears to induce the full loss of exocrine tissue secretory function. In this regard, the B cell-deficient NOD mouse represents an advance in our ability to evaluate the role of specific immune cells in

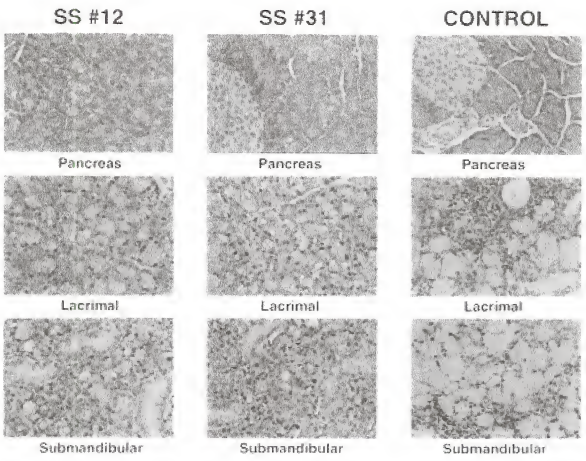


Fig. 2-4. Histologic profiles of the exocrine tissues of NOD.Igμ^{null} mice after chronic treatment with control or Sjögren's syndrome IgG fractions. The sections of submandibular, lachrymal, and pancreas were stained by hematoxylin/eosin and observed by light microscopy (400X). Sjögren's syndrome patients #12 and 31, one stimulating secretion and the other inhibiting secretion, were used in this evaluation.

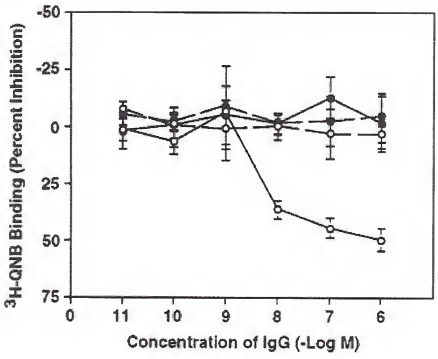


Fig. 2-5. Inhibition of [^{3}H]QNB binding to NOD parotid gland membranes by primary Sjögren's syndrome IgG (n = 5; open circles) or normal patient IgG (n = 3; solid circles). Increasing concentrations of human IgG were incubated with parotid gland membranes followed by the addition of 0.5 nM radiolabeled ligand agonist (21) (solid lines). Immunoprecipitation of [^{3}H]QNB -labeled muscarinic receptors by normal and patient IgG is represented by the dashed lines. The results are the mean ± SE for Sjögren's syndrome patients (#36, 71, 77, 93, 97) and normal healthy controls (#4, 5, 6) performed on two separate occasions in duplicate. Control binding of 100% refers to the value of [^{3}H]QNB bound to the membrane without the addition of antibody reagents (20).

exocrine tissue secretory function.

Salivary flow is a result of neural stimulation of the acinar and ductal cells of the glands, specifically in response to muscarinic/cholinergic receptor agonists. In contrast, the protein phase is generated in response to agonists stimulating the β-adrenergic receptors.[117,118,119] Autoantibodies directed against autonomic nervous system receptors and possessing agonistic properties have been described in a number of human pathologies.[120,121] Our previous studies in NOD mice have shown decreased receptor density and intracellular signal transduction components, along with autoantibody populations directed against the β-adrenergic and muscarinic/cholinergic receptors.[69,113] Alterations also have been detected in the glandular innervation of the exocrine tissues from Sjögren's syndrome patients.[122,123] Recent analyses of autoantibodies from primary Sjögren's syndrome patients have detected anti-muscarinic receptor activity capable of acting as a neural receptor agonist.[71,124,125]

Although the present study does not exclude a role for other circulating autoantibodies, such as SS-A or SS-B,[100,101] in mediating hypofunction, anti-neural receptor antibodies represent an intriguing potential mechanism for the down-regulation of exocrine secretion. Desensitization of the signal-transduction response can occur through chronic activation of agonist-binding sites or through the generation of autoantibodies capable of directly blocking or sterically hindering the ability of agonists to bind to cell surface receptors, thereby depriving cells of the necessary signals to generate a secretory response. Alternatively, chronic stimulation of receptors can lead to desensitization of the secretory response through down-regulation of surface receptor density.[126]

The ability of IgG and $F(ab')_2$ fragments of sera from SS patients to either stimulate or inhibit secretory function could be related to the stage of the disease at the time the serum was collected or the titer of specific autoantibody fractions. In our study, we found evidence of both exocrine stimulation and inhibition in different Sjögren's patient IgG. One possible explanation for our results would be that the anti-glandular reactive antibody titer is important in determining whether the IgG is stimulatory or inhibitory in NOD.Igμ^{null} mice. We have not observed a similar response when IgG from systemic lupus erythematosus or rheumatoid arthritis patients, free of secondary Sjögren's syndrome complications, are injected into NOD-scid mice (unpublished observations). The ability of a subset of Sjögren's syndrome patients to stimulate secretory response is intriguing in light of the potential therapeutic value to patients suffering from secretory dysfunction.

Histological analysis of exocrine tissues treated with patient IgG or $F(ab)_2$ fractions revealed marked morphological changes. Subsequently, both terminal deoxynucleotidyl transferase-mediated UTP end labeling and a specific caspase-3 assay have been conducted to determine whether increased programmed cell death (PCD) is related to this observation. Preliminary results have provided no evidence for increased PCD after antibody transfer (J.B., A.B.P., and M.G.H.-B., unpublished observations). An alternative explanation to PCD is that the autoantibody reaction with receptors on the exocrine cell surface provokes a functional quiescence in the cells manifested in loss of stimulated secretory response. The appearance of histologically identifiable vacuolar structures may be the result of fusion of intracellular secretory granules and digestion of their contents because of a lack of response. Thus, by entering a period of functional rest, the cells may

avoid further attack by the activated immune system. The induction of functional quiescence is consistent with the observation that the salivary glands are able to fully recover secretory response after a 1-week washout period in which antibody treatment is terminated.

In summary, the results presented here indicate a primary role for autoantibodies, presumably directed against the autonomic nervous system receptors[69,71,113] as playing a primary role in the clinical manifestation of Sjögren's syndrome, namely, the loss of secretory function. The identification of these antibodies as agents in the disease process suggests new modalities for treatment potentially based on specific immunomodulatory therapy.

CHAPTER 3
CHARACTERIZATION OF THE NOD.IL-4$^{-/-}$ AND NOD.IFN-$\gamma^{-/-}$ MICE

Introduction

While more commonly studied as a model of autoimmune diabetes, the NOD mouse has emerged as an appropriate model for autoimmune exocrinopathy, paralleling the human autoimmune disease, Sjogren's syndrome (SS).[22] As in the human disorder, these mice develop focal lymphocytic infiltrates in the salivary and lachrymal glands leading to the eventual loss of secretory capacity in these exocrine tissues. Studies in the NOD-*scid* congenic derivative strain have shown that, in the absence of an adaptive immune response, the salivary glands still undergo dramatic morphological rearrangement typified by a loss of the majority of the secretory acinar cell population and a replenishment, or hyperproliferation of the ductal epithelial cells.[24] Despite the deterioration of the secretory cells, these animals maintain normal salivary function throughout life, suggesting that the remaining acinar cells are capable of compensating for lost secretory capacity. More important, though, this animal model highlights the requirement for the active adaptive immune response in the loss of glandular function. More recent studies in the NOD.Igμ^{null} mouse, another congenic strain which specifically lacks B lymphocytes, reveal that these animals also maintain normal salivary secretory capacity, indicating the importance of the B cell in driving secretory hypofunction.[127] Furthermore, the transfer

of human IgG purified from the serum of SS patients, or IgG purified from NOD mice exhibiting SS-like symptoms, induced a dramatic reduction in secretory capacity of recipient NOD.Igμ^{null}, NOD-*scid*, or C57BL/6-*scid* mice within 24 hours, an effect which could be sustained through the administration of additional doses of IgG. These effects could not be duplicated using serum IgG from healthy donors or control strain mice such as Balb/c or C57BL/6. These observations conclusively demonstrate that antibodies associated with SS are driving the loss of function.

SS is classified as a chronic systemic autoinflammatory disorder primarily affecting the salivary and lachrymal glands. It is further characterized as a lymphoproliferative disorder associated with elevated titers of IgM and IgG, and numerous autoantibodies have been identified over the past 25 years. The first autoantibodies identified were directed against the nuclear antigens Ro/SS-A and La/SS-B,[63] although additional studies have not yet shown a functional relevance of these specific autoantibodies to the disease. As a result, they have been considered important markers for the disease and, as such, have been included as part of the diagnostic criteria for SS. In the last decade, several novel autoantibodies have been discovered in the study of SS, recognizing a variety of antigens including parotid secretory protein (PSP),[66] carbonic anhydrase,[67] α-fodrin,[68] β-adrenergic receptor,[113] and the muscarinic receptor.[69,70] While none of these species has yet been directly correlated with a decrease in function in the exocrine glands, it is interesting to consider that the β-adrenergic and muscarinic receptors are primarily responsible for the protein and fluid phases, respectively, of tears and saliva.[128] Additionally, the presence of the autoantibodies against the nuclear antigens and, quite possibly, other intracellular antigens such as α-fodrin may be indicative of autoreactivity

against apoptotic cells.[94] With the current observations from the studies in the NOD.Igμ^{null} mice, attention is turning once again to these autoreactive antibodies in attempts to isolate specific effector antibodies and determine mechanisms of activity.

The production of antibodies in any immune response is regulated by the cytokines expressed in the lesion and draining lymphatics. In efforts to further elucidate the roles of specific cytokines in the pathogenesis of autoimmune diabetes, genetic knockouts of IFN-γ,[129] interleukin-4 (IL-4),[130] and IL-10 have been introduced into the parental NOD mouse strain. IFN-γ is considered a central cytokine in the promotion of a T_H1 inflammatory response, whereas IL-4 and IL-10 are considered T_H2-type cytokines. Therefore, the deficiencies of any of these cytokines could alter the normal progression of the immune response and impact the disease pathogenesis profoundly.

The ability to modulate the autoimmune response through the therapeutic use of cytokines may provide new avenues for intervention and amelioration of the underlying pathology. In the case of diabetes, IL-4 does not appear to alter the disease onset;[130] however the lack of IFN-γ seems to delay but not prevent onset.[129] The impact of IL-10 in diabetes currently has not been reported using the NOD.IL-10$^{-/-}$ mouse model to date; however, multiple studies measuring the influence of IL-10 through the administration of IL-10 either by injection or through transgenic expression suggest that this cytokine can both exacerbate and protect against diabetes onset depending on the timing.[131] The impact of these cytokine deficiencies on the sialoadenitis in these mice has not been ascertained. More importantly, as these cytokines are critical in shaping the antibody responses of the adaptive immune system, these mice may be able to provide important insights into the identity, as well as the mechanisms of development and activity of the

effector autoantibodies driving the glandular hypofunction associated with autoimmune exocrinopathy.

The scope of this present study was to begin to characterize these cytokine-deficient NOD congenic strains with an emphasis on the development of SS-like pathology. Animals were analyzed for the development of the primary traits of autoimmune exocrinopathy, including histological, physiological, serological and biochemical markers of disease. The observations presented in this work suggest that both IFN-γ and IL-4 but not IL10 play important roles in the onset of functional suppression of the salivary glands.

Materials and Methods

Materials. NOD.IL-4$^{-/-}$, NOD.IL-10$^{-/-}$ and NOD.IFN-γ$^{-/-}$ mice were a kind gift from Dr. David Serreze, Jackson Laboratories (Bar Harbor, ME) and were bred and maintained under specific pathogen-free conditions in the mouse facility at the University of Florida, along with Balb/c, C57BL/6, NOD/Lt and NOD.Igμ^{null} mice. Both male and female mice were used at three time points, 8, 12, and 20 weeks.

Saliva collection and preparation of gland lysates and histological sections. Saliva was collected following stimulation of secretion using isoproterenol (0.20mg/100g of body weight) and pilocarpine (0.05mg/100mg body weight) dissolved in saline as described previously.[22,106] Saliva samples were collected over a 10 minute period and frozen at -80^{O}C for further analysis. Excised lachrymal, submandibular, parotid and pancreas were fixed in 10% neutral buffered formalin for 24 hours in preparation for paraffin embedding for histological sections, or were homogenized in 10mM Tris buffer (pH 7.4) and immediately frozen at –80^{O}C. Protein concentrations of saliva and gland

lysates were determined using the method of Bradford.[132] Histological sectioning and staining with Mayer's hematoxylin and eosin was performed by the University of Florida Diagnostic Referral Laboratories, Gainesville, FL.

α–Amylase Analysis. Amylase was determined by its ability to hydrolyze starch according to published protocols.[133] In brief, 500- to 1000-fold dilutions of saliva in PBS were added to a solution containing 0.4g soluble starch in 60mM Tris-HCl, 0.15M NaCl, and 3mM $CaCl_2$. The reaction was stopped after 5 or 10 minutes by the addition of 0.045% I_2, 0.045% KI, and 0.03N HCl. Absorbance was measured at wavelength of 620nm. One unit of amylase was defined as the amount that hydrolyzed 1mg of starch/min/mg protein at 37^{O}C.

Polyacrylamide gel electrophoresis and Western blot analysis. Total salivary proteins (15 μg/well) or gland lysates (50 μg/well) were separated on 10% or 12% sodium dodecyl sulfate-polyacrylamide gels.[134] The proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA)[135] and immunoblotted with a rabbit polyclonal antibody to rat PSP at a 1:10,000 dilution.[66,136,137] The blocking buffer consisted of 3% nonfat fry milk and 3% bovine serum albumin in Tris-buffered saline. Following 3 10-minute washes, the membranes were incubated with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin and exposed to substrate as previously described.[24]

Anti-nuclear antibody (ANA) staining. Sera from NOD, Balb/c, NOD.Igμ^{null}, NOD.IL-4$^{-/-}$, NOD.IL-10$^{-/-}$, and NOD.IFN-γ$^{-/-}$ mice were collected by femoral bleed. Detection of ANA was accomplished with an ANA kit (Sigma, St.Louis, MO) using human hepatocytes.[22] A 1:80 dilution of mouse serum was incubated for 3 hours with

cells and then with a fluorescein isothiocyanate-conjugated goat anti-mouse antibody. Nuclear fluorescence was detected by fluorescence microscopy using standard procedures.

Terminal UTP nucleotide end labeling (TUNEL) histological assay. Paraffin-embedded submandibular tissues were cut into sections of 5μm thickness and deparaffinized. Sections were hydrated in 2 washes in xylene followed by successive 5 minute washes in 100% and 95% ethanol, and finally in two 1 minute washes of 70% ethanol, and then equilibrated in PBS (pH 7.4). Tissues were then partially digested for 20 minutes with proteinase-K (20μg/ml), and endogenous peroxidases were inactivated following this with a 5 minute incubation in 3% H_2O_2. DNA fragmentation was detected using an Apoptag Kit (Oncor, Gaithersburg, MD), which labels 3' ends of DNA with a digoxigenin-conjugated dUTP and dATP using terminal deoxynucleotide transferase (TdT).[138] Histological sections were allowed to incubate with the digoxigenin dNTP's for 1 hour at 37^{O}C in the presence of TdT. Labeled DNA was detected using a peroxidase-conjugated goat anti-digoxigenin antibody, applied to the histology sections for 30 minutes, followed by exposure to the chromogenic substrate diaminobenzidine with 0.02% H_2O_2. Sections were counterstained with methyl green for contrast. Stained sections were analyzed under a light microscope.

Statistical analysis. All measures of variances are given as standard deviations of the mean. Tests of significance for differences between independent means were performed with unpaired Student's *t* test. Results in which $P < 0.05$ were considered significant.

Results

Salivary function. The NOD mouse stands apart from most other animal models of SS in that it exhibits an immune mediated loss of secretory function.[22,24] Therefore, the characterization of the IL-4-, IL10- and IFN-γ-deficient NOD congenic strains began with the evaluation of secretory capacity at 8, 12, and 20 weeks of age. These ages represent a pre-disease point, the time when the initial development of exocrine gland focal lymphocytic infiltration occurs, and a time point corresponding to the onset of an acute disease state associated with secretory hypofunction, as previously demonstrated.[52] The NOD.Igμ^{null} animal has established a case for the role of autoantibodies in the loss of function;[127] while cytokines, and especially IFN-γ, have been shown to exert strong influences and even cytotoxic effects on salivary epithelial cell line.[61,62] Figure 3-1 shows the results of the evaluation of saliva volumes generated following stimulation over a 10 minute period from each strain at the indicated ages (n = 6-8 per group). Results of the functional analyses of these animal strains reveal that the absence either of the prototypic T_H1 cytokine, IFN-γ or the prototypic T_H2 cytokine, IL-4, profoundly alters disease progression such that neither strain loses secretory function. More specifically, in the absence of IFN-γ the functional levels were similar to those found in the NOD-*scid* and NOD.Igμ^{null} mice while the NOD.IL-4$^{-/-}$ mice exhibited a slight decrease in secretory capacity as compared to the immuno-deficient strains, but this was not statistically significant and was not nearly as pronounced as that observed in the NOD animal. The lack of IL-10 in the genetic context of the NOD mouse appeared to exert little effect on the gland function, as these animals developed all the hallmark manifestations of autoimmune exocrinopathy (loss of secretory function, focal

lymphocytic infiltration, serum anti-nuclear autoantibodies) as observed in the NOD parental strain.

Histological evaluation of the salivary and lachrymal glands. A second important hallmark of SS and the SS-like disease in the NOD mouse is the development of focal lymphocytic infiltrates in the exocrine tissues. Based on the studies in the NOD and NOD-*scid* model system, the development of focal infiltrates is closely related to the onset of secretory suppression. Histological samples were collected from the three cytokine-deficient strains at 20 weeks to assess the appearance of lymphocytic foci in the submandibular and lachrymal glands (parotid glands rarely develop focal infiltrates in the NOD mouse) (Fig. 3-2). Glands from NOD and C57BL/6 mice were evaluated as positive and negative controls, respectively. Once again, the NOD.IL-$10^{-/-}$ exhibited normal pathological progression, showing initial signs of glandular infiltration by 12 weeks and heavy infiltration by 20 weeks of age. The NOD.IL-$4^{-/-}$ mouse exhibited signs of exacerbated inflammation of the exocrine tissues, with infiltrates detected at the 8 week time point in some cases. By 20 weeks of age, the salivary glands exhibited severe infiltration, with the appearance of massive peri-ductal foci exceeding the size typically found in the NOD mouse by 2- to 3-fold. Severity of inflammation was also assessed in terms of frequency of focal infiltration of the salivary glands, as determined by counting the number of lymphocytic foci per $4mm^2$ tissues section, and these results support the assessment that the NOD.IL-$4^{-/-}$ exocrine glands appear to undergo a more intense inflammatory attack. In contrast, the NOD.IFN-$\gamma^{-/-}$ mouse showed no signs of focal infiltrates in the salivary glands even at the 20-week time point (sparse infiltrates in the submandibular gland were occasionally detected at >30 weeks of age). Furthermore, the

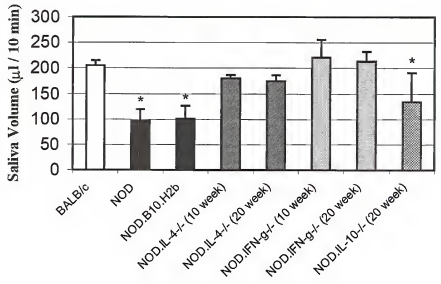


Figure 3-1. Histogram of saliva production in cytokine-deficient NOD congenic mice. Saliva was collected for 10 minutes from BALB/c, NOD, NOD.IL-4$^{-/-}$, NOD.IL-10$^{-/-}$, and NOD.IFN-$\gamma^{-/-}$ mice following stimulation of salivation by intraperitoneal injection of isoproterenol and pilocarpine. (* $P < 0.05$)

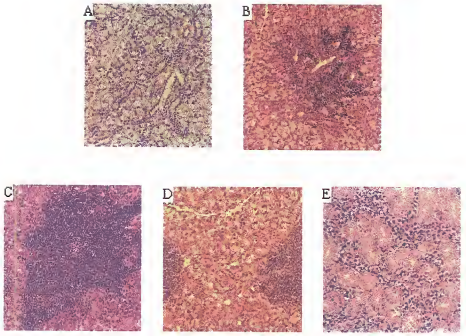


Figure 3-2. Histological analysis of the submandibular glands. Lachrymal glands were excised from 20 week old C57BL/6 (*A*), NOD (*B*), NOD.IL-4$^{-/-}$ (*C*), NOD.IL-10$^{-/-}$ (*D*), and NOD.IFN-$\gamma^{-/-}$ (*E*) mice. Focal lymphocytic infiltrates were identified as aggregates of >50 lymphocytes.

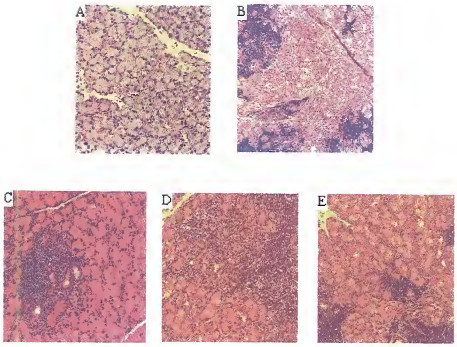


Figure 3-3. Histological analysis of the lachrymal glands. Lachrymal glands were excised from 20 week old C57BL/6 (*A*), NOD (*B*), NOD.IL-4$^{-/-}$ (*C*), NOD.IL-10$^{-/-}$ (*D*), and NOD.IFN-γ$^{-/-}$ (*E*) mice. Focal lymphocytic infiltrates were identified as aggregates of >50 lymphocytes.

submandibular glands of these mice appeared to maintain a greater acinar cell population than any other NOD strains, including the NOD-*scid*,[22,23,24] suggesting that, while the antibodies may be the major direct mediators of functional suppression, IFN-γ may play a critical role in the tissue rearrangement and acinar cell loss observed in SS and the mouse models of the disease. All strains, including the NOD.IFN-$\gamma^{-/-}$ exhibited severe lymphocytic infiltration of the lachrymal glands by 20 weeks of age (Fig. 3-3).

Detection of ANA in serum. The third major criteria for the diagnosis of SS relies on the serological detection of ANA's or rheumatoid factor (rf-IgM). While in the NOD mouse, rf-IgM has not been detected, analysis of NOD serum in animals exhibiting overt exocrinopathy reveals the presence of ANA.[23] We therefore tested whether antibodies in the serum of these congenic mice could recognize nuclear components of HepG-2 cells. Antibodies from IL-4-, IL-10- and IFN-γ-deficient mice as well as positive control NOD, and negative control Balb/c and NOD.Igμ^{null} mice were diluted 1:80 and incubated with HepG-2 cells fixed to slides. Presence of ANA was performed by indirect immunofluorescence. Results from this assay indicate that NOD.IFN-$\gamma^{-/-}$ mice do not develop ANA, whereas both the NOD.IL-$4^{-/-}$ and NOD.IL-$10^{-/-}$ possess ANA in their sera by 20 weeks of age (Table 3-1).

Biochemical analysis of saliva. In addition to the rheumatological aspects of this autoimmune exocrinopathy, NOD mice also develop several biochemical features associated with the disease progression. As the animals begin to experience decreases in saliva volume, the protein concentrations of the saliva produced increases, although not directly in proportion to the lack of fluid. Along with this change saliva protein concentration, there are also several measurable alterations in protein composition that

can be detected both in the saliva and in salivary gland homogenates. There is an appreciable decrease in α-amylase and EGF activity[106] over time, and additionally, there is a reappearance of PSP in the submandibular gland in an aberrant form, missing the first 20 amino acids of the protein.[24] As this novel cleavage product begins to be detected, a novel protease can also be detected in the saliva and gland lysates of the mice indicating that this aberrant form of PSP is the result of post-translational processing.[24]

All three cytokine-deficient NOD strains evidence significant biochemical changes that can be detected in accordance with the SS-like pathology found in the NOD mouse. Aberrant PSP expression was detected as early as 10 weeks of age in the NOD.IL-$4^{-/-}$, NOD.IL-$10^{-/-}$, and NOD.IFN-$\gamma^{-/-}$ mice (Fig. 3-4). Additionally, the three congenic strains demonstrated a decrease in α-amylase activity over time as compared to control BALB/c strains which maintain enzyme activity levels (Table 3-2). Assays for total protein concentration of the saliva of the IL-4-, IL-10-, and IFN-γ-deficient strains revealed that, as with the NOD parental strain and in contrast to control strain saliva, the protein concentrations increased with age in accordance with disease onset (Table 3-2).

Analysis of apoptotic activity via TUNEL staining. In the last few years, apoptosis has been shown to play an important role in the onset of SS and in the murine form of autoimmune exocrinopathy.[94,139] In the NOD mouse, increases in apoptotic activity can be detected in the affected glands by 20 weeks of age as compared to the activity found in the glands of Balb/c control mice or in 8-week tissue samples from NOD mice.[93] Interestingly, this activity can also be detected in the glands of 20-week NOD-*scid* mice in the absence of any immune infiltrates.[94] Levels of apoptosis have been established according to several indicators, which include the induction of Fas expression in the

exocrine epithelial cells,[86] and the expression or activation of bax and caspase-3 in these cells.[99] The measurement of cysteine protease activity in the gland homogenates and saliva,[94] and the detection of apoptotic cells immunohistochemically via TUNEL staining.[95]

With respect to these cytokine-deficient congenic NOD strains, the NOD.IL-$10^{-/-}$ mice appear to develop all the typical hallmarks of the SS-like pathology, while the IL-4- and IFN-γ-deficient NOD mice both seem to be impaired with respect to the development of the autoimmune process to differing degrees. Furthermore, the NOD.IFN-$\gamma^{-/-}$ mouse does not appear to develop significant salivary pathology. Therefore, we focused on the NOD.IL-$4^{-/-}$ and NOD.IFN-$\gamma^{-/-}$ mice to explore in more depth the correlation between the induction of apoptosis and gland histopathology and secretory dysfunction. Measurements of apoptotic activity were assessed as the number of TUNEL-staining cells per $4mm^2$ section of tissue. TUNEL analysis of 12-week and 20-week histological sections reveal that, despite the increase in inflammation in the glands, there appears to be similar levels of apoptotic activity in the NOD.IL-$4^{-/-}$ to that observed in the NOD-derived salivary sections, although both exhibit slightly increased numbers of apoptotic cells as compared to the control C57BL/6 strains (Fig. 3-5). The NOD.B10.$H2^b$ and NOD.IL-$4^{-/-}$ strains both exhibit statistically significant differences from the control strain at both 12 and 20 week time points. Conversely, the NOD.IFN-$\gamma^{-/-}$ mouse appears to exhibit fewer apoptotic events per mm^2 of histological section than the NOD or NOD.IL-$4^{-/-}$ mice, with apoptotic activity measuring much more closely to the levels found in the control strains.

	12 week	20 week
NOD	No	Yes
BALB/C	No	No
NOD.IL-4$^{-/-}$	No	Yes
NOD.IL-10$^{-/-}$	No	Yes
NOD.IFN-$\gamma^{-/-}$	No	No

Table 3-1. Detection of antinuclear antibodies in mouse serum. Serum from 12 and 20 week old NOD (*A* and *B*), BALB/c (*C* and *D*), NOD.IL-4$^{-/-}$ (*E* and *F*), NOD.IL-10$^{-/-}$ (*G* and *H*), and NOD.IFN-$\gamma^{-/-}$ (*I* and *J*) mice were diluted 1:40 in PBS and reacted with HepG2 cells. Preparations were washed and then incubated with a FITC-conjugated goat-anti-mouse IgG secondary antibody and visualized using a fluorescent scope.

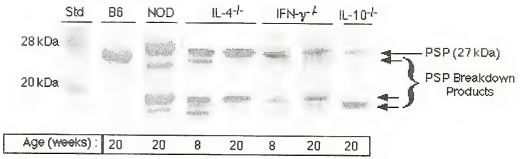


Figure 3-4. Western blot analysis of PSP in the saliva of cytokine-deficient NOD congenic strains. Saliva from a 20 week old BALB/c mouse was used as a negative control and saliva from a 20 week old NOD mouse served as a positive control. PSP appears as a 28kDa band while aberrantly cleaved PSP or breakdown products are detected as smaller fragments and are labeled in the figure.

Table 3-2. Biochemical analysis of saliva from the cytokine-deficient NOD congenic strains.

		Protein Concentration (mg/ml)	α-Amylase Activity (mg starch/min/mg prot)
NOD	(10 week)	N	N
	(20 week) [a]	5.98 ± 0.11	362.
BALB/c	(10 week)	N	N
	(20 week) [a]	3.75 ± 0.6	557.
NOD.I -4$^{-/-}$	(10 week) [b]	4.07 ± 0.29	424.1
	(20 week) [b]	3.02 ± 0.13	479.
NOD.I -10$^{-/-}$	(10 week)	N	N
	(20 week) [b]	3.73 ± 0..35	351.6
NOD.IF -$\gamma^{-/-}$	(10 week) [b]	5.43 ± 0.23	315.1
	(20 week) [b]	4.38 ± 0.25	426.9

[a] values were derived from previous studies of the NOD mouse (8,9,17,18)

[b] calculations are based on an n = 5

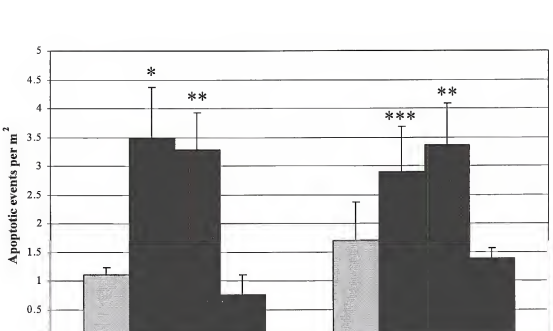


Figure 3-5. TUNEL analysis of submandibular glands of NOD.IL-4$^{-/-}$, NOD.IL-10$^{-/-}$, and NOD.IFN-$\gamma^{-/-}$ mice. Histological sections were prepared from NOD, C57BL/6 (CTL), NOD.IL-4$^{-/-}$(IL-4$^{-/-}$), and NOD.IFN-$\gamma^{-/-}$ (IFN-$\gamma^{-/-}$) mice of 12 and 20 weeks of age. Evaluation of stained sections was performed and values are reported as the number of stained acinar cells per mm^2 of tissue section. Statistically significant differences between Control and congenic strains were determined by the Student's t test (*P>0.01, **P>0.005, ***P>0.02).

Discussion

The development of autoimmune exocrinopathy in the NOD mouse results in a condition that closely mimics the human disease, Sjögren's syndrome. Through observations in the NOD mouse and in the congenic derivative, the NOD-*scid*, the underlying events that drive the onset of the disease have been attributed to a combination of genetic or developmental and immunological factors that act in concert to bring about the lymphocytic infiltration of the salivary and lachrymal glands and resultant loss of secretory function. While the dramatic morphological rearrangement of the salivary glands, consisting of a loss of acinar cells and a hyperproliferation of ductal cells, occurs in the absence of lymphocytic presence, as revealed in the NOD-*scid*, the loss of secretory capacity is dependent on the activity of the immune infiltrates, and, more specifically, the infiltrating B cells and the antibodies they produce, as evidenced by the normal salivary function in the NOD.Igμ^{null} mouse.[127] Despite the direct correlation between the loss of function and presence of autoantibodies,[127] there are still important questions to be resolved concerning the underlying immune response that eventually leads to the production of these pathogenic autoantibodies. Using the NOD congenic mice exhibiting deficiencies in IFN-γ, IL-4, and IL-10, this work begins to address the importance of the T_H1-type and T_H2-type cytokines in shaping the ensuing immune response in the exocrine tissues.

The function of IL-10 has been a point of interest in the study of SS for several reasons. First of all, in light of the classical characterization of SS as an autoinflammatory disorder, IL-10 has been a cytokine of interest due its anti-inflammatory properties, tending to suppress cell proliferation and macrophage

activation. In apparent contrast to this generalized characterization, this cytokine is detected at relatively strong expression levels in the exocrine lesions in both the human and murine diseases.[52,54,55,56] Thus, it is interesting to note that the absence of this cytokine does not affect disease onset in the NOD background. This is even more surprising considering the recent evidence that transgenic expression of IL-10 under the salivary amylase promoter, generating constitutive expression of this cytokine in the submandibular gland in a C57BL/6 mouse strain, can cause a SS-like disease including a decrease in salivary function.[140] However, this should not necessarily be interpreted as a contradiction, but rather as a prime example of the importance of timing of expression as opposed to strict cytokine presence or absence in directing an immune response. Furthermore, the authors of this IL-10 transgenic expression work assert that the introduction of IL-10 leads to an induction of FasL-mediated apoptosis that leads to autoimmune exocrinopathy. This would be consistent with our findings in the NOD mouse in that, even in the absence of constitutively expressed IL-10, there is excessive apoptosis[94] in the glands that could initiate the autoimmune pathogenesis.

The observed changes in disease pathogenesis in the NOD.IL-4$^{-/-}$ and NOD.IFN-$\gamma^{-/-}$ mice, however, are much more interesting. First of all, in the absence of IFN-γ, there appears to be little if any signs of the salivary aspects of autoimmune exocrinopathy. Mice maintain salivary capacities throughout life and there are very few indications of glandular disturbances histologically. Beginning at approximately 30 weeks, these mice develop sparse focal infiltrates, although there is no corresponding loss of function. Quite possibly, these infiltrates are not related to the incidence of autoimmune exocrinopathy found in the NOD background but are simply a reflection of the ongoing

autoimmune response in the pancreas of these animals, which develops around this time in the life of the animal.[129] Perhaps the most interesting observation in the NOD.IFN-$\gamma^{-/-}$ mouse is the fact that the submandibular glands retained the acinar cell population to a much greater degree than the NOD and even the NOD-*scid* mouse. The loss of the acinar population even in the absence of focal lymphocytic infiltration of the gland led to the conclusion that the dramatic tissue morphological rearrangement was a consequence of an underlying glandular defect that drew the progressive autoinflammatory response. The characterization of the NOD.IFN-$\gamma^{-/-}$ mouse forces us to reexamine that conclusion and entertain the hypothesis that, while biochemical analyses reveal potentially disruptive disturbances in the salivary glands in the absence of an immune presence, the disappearance of acinar cells may be a result of the presence of elevated or persistent levels of IFN-γ in the gland, through potential mechanisms such as alternate antigen processing or the upregulation of HLA expression. Certainly, this deserves future attention in light of the fact that *in vitro* studies have demonstrated the susceptibility of glandular epithelial cells to cytotoxic effects of IFN-γ.[61]

The INF-γ gene knockout mouse also begins to reveal an important distinction between the lachrymal and salivary pathologies. While the salivary aspects of the autoimmune exocrinopathy barely appear, the lachrymal glands of these mice become heavily infiltrated by 20 weeks of age. Functional assays have not yet been performed, nor have the infiltrating cells been characterized. However, the histology indicates that there are important distinctions in the conditions that allow or promote the autoimmune attack between the salivary and lachrymal glands. Interestingly, the NOD mouse was originally created as a model for glaucoma. Thus, the picture from the NOD.IFN-$\gamma^{-/-}$

mouse may also be suggesting that the SS-like disease in these animals may initiate in the lachrymal glands, or, alternatively, that, just as the pathological condition in the NOD mouse has been segregated into two separate diseases, autoimmune diabetes and autoimmune exocrinopathy, the SS-like disorder may need to be viewed as separate but interrelated oral and ocular pathologies.

The repercussions from the IL-4 deficiency in the NOD mouse has been, arguably, the most interesting of the three cytokines analyzed with respect to the immune-mediated loss of function. Most importantly, this animal demonstrates conclusively that, while IL-4 is rarely if ever detected in the exocrine tissues, it is nonetheless essential in the progression from the initial autoimmune infiltration of the gland to the functionally debilitating stages of the disease. This finding, alone, suggests that the classification of this disease as an autoinflammatory disorder (coincidently due, in part, to the lack of IL-4 expression in the infiltrates in the affected glands) should be reconsidered. This is not to suggest an insignificance of the inflammatory infiltrates, as the NOD.IFN-$\gamma^{-/-}$ stresses the critical role of this inflammatory cytokine on the development of most hallmark aspects of the disease. This IL-4-deficient congenic animal model lends further support to the increasing emphasis on the humoral component to this disease rekindled by the realization that the loss of secretory function is mediated by autoantibodies.[127]

What makes the NOD.IL-$4^{-/-}$ mouse most interesting is that the animal develops more severe inflammation of the salivary glands, compared to the NOD mouse, produces ANA, and yet, does not lose salivary function. These observations suggest two important conclusions. Connections have been established previously between the loss of function and the presence of serum autoantibodies, however, the predominance of T_H cells and

high expression levels of IFN-γ known to exhibit cytotoxic effects on salivary epithelial cells had supported the hypothesis that cytokine-mediated destruction of the secretory cells contributes to the loss of function. First of all, this animal model provides strong contradictory evidence against the established hypothesis of the role of the inflammatory lesions as a direct mediator of secretory dysfunction. Secondly, this model distinguishes the presence of ANA's as functionally separate from the subset of autoantibodies responsible for the loss of glandular function. Further analysis of the immune response in the NOD.IL-$4^{-/-}$ mouse should contribute to the future identification of functionally relevant autoantibodies and potentially the important events in the disease pathogenesis leading to their generation.

Altogether, the implications derived from the characterization of the NOD.IL-$4^{-/-}$ and NOD.IFN-$\gamma^{-/-}$ mice suggest that the development of autoimmune exocrinopathy in the NOD mouse relies on an initial development of an inflammatory infiltration of the gland and a secondary humoral response that more directly hinders gland function. More importantly, both are essential to the development of secretory hypofunction. Thus, SS cannot truly be classified as either a T_H1- or a T_H2-mediated autoimmune disease.

CHAPTER 4
IMPACT OF CYTOKINE DEFICIENCIES ON DISEASE DEVELOPMENT

Introduction

Sjögren's syndrome (SS) is a chronic systemic autoimmune disorder primarily affecting the salivary and lachrymal exocrine glands and resulting in a reduction in tear and saliva secretion. Histological examination of the glands reveals the presence of focal lymphocytic infiltrates, aggregates of 50 or more lymphocytes, which typically form in peri-ductal regions of the glands. Further characterization of these populations of immune cells reveals that these infiltrates consist predominantly of $CD4^+$ $\alpha\beta$ T cells, although there are also distinct subpopulations of both B cells and $CD8^+$ T cells. Cytokine expression profiles have been measured in terms of both mRNA and protein levels,[54,55,58] and both suggest an increase in IL-1β, IL-6, IL-10, IL-12, IFN-γ, and TNF-α. Together, these traits of the immune presence in the exocrine tissues lead to the widely accepted conclusion that SS is an inflammatory disorder.

SS has also been described as a lymphoproliferative disorder, associated with hypergammaglobulinemia. Numerous autoantibodies have been detected in patient serum, including those against such antigens as the nuclear proteins Ro/SS-A and La/SS-B,[63] parotid secretory protein (PSP),[66] carbonic anhydrase,[67] α-fodrin,[68] and the

muscarinic receptor.[69] Recent studies in the NOD.Igμ^{null} mouse, a congenic derivative of the NOD model of SS, have demonstrated the importance of these antibodies in the loss of glandular function. Furthermore, a disruption of gland function can be reconstituted in these animals by passive transfer of serum IgG from either NOD mice or human SS patients.[127] Thus, there is a critical dependence on autoantibodies for the induction of the most common overt symptoms of the disease, oral and ocular dryness.

The discovery that autoantibodies drive the loss of function leads to an important question as to whether there is an underlying T_H2 response contributing to the pathogenesis of SS. This is potentially highly relevant to the mechanism by which these antibodies are influencing exocrine function. The characterization of the NOD.IL-4$^{-/-}$ and NOD.IFN-γ$^{-/-}$ mice has lent further support to the existence of an underlying humoral response in the disease pathogenesis. While the inflammatory cytokine IFN-γ appears to act more globally on the disease state, the T_H2-type cytokine IL-4 seems to effect more precisely the development of glandular hypofunction. Interestingly, the NOD.IL-4$^{-/-}$ animal maintains the capacity to produce anti-nuclear antibodies (ANA), suggesting that they are affiliated somehow with the inflammatory aspects of the disease, which persists in the NOD.IL-4$^{-/-}$ background. This supports the notion that the loss of secretory capacity is the result of antibodies generated in a secondary humoral phase of the SS pathology.

Just as the NOD.IL-4$^{-/-}$ mouse has provided significant evidence to support the theory that loss of secretory capacity associated with autoimmune exocrinopathy is a function of a humoral immune response, the NOD.IFN-γ$^{-/-}$ mouse has also challenged the current paradigm of disease development in the NOD mouse and presumably in SS patients. In

the characterization of the NOD-*scid* mouse, the progression of gross morphological rearrangement of the submandibular cell components in the absence of lymphocytic infiltration led to the conclusion that the submandibular glands exhibit a developmental or genetic defect that predisposes and contributes to the generation of an immunological attack on the glands. Retention of the acinar cell population in the NOD.IFN-$\gamma^{-/-}$ mouse presents a potential contradiction to this proposed model, suggesting that INF-γ, known to have cytotoxic effects on salivary epithelial cells,[141] could be mediating the destruction of the acinar cell population, rather than an unidentified glandular genetic defect.

In this study, we have focused primarily on the development of SS in NOD.IFN-$\gamma^{-/-}$ and NOD.IL-$4^{-/-}$ mice as a means to further dissect the immunological attack on the exocrine glands. The impact of the cytokine deficiencies was assessed in terms of intraglandular cytokine production profiles, immune cell distribution in the focal lymphocytic infiltrates, and serum IgG subclass production and distribution. Results suggest that, in the development of SS-like lesions in the NOD mouse, IFN-γ plays an essential role in morphological rearrangement leading to focal inflammation, while the B cell may potentially serve a more instructive role in the pathogenesis, acting as an antigen-presenting cell in addition to producing the antibodies that drive the loss of function.

Materials & Methods

Animals. NOD.IL-$4^{-/-}$ and NOD.IFN-$\gamma^{-/-}$ mice were a kind gift from Dr. David Serreze, Jackson Laboratories, as were the NOD.IL-$10^{-/-}$ mice included in the IgG subclass analysis. These strains were bred and maintained under specific pathogen-free conditions in the mouse facility at the University of Florida, along with Balb/c, C57BL/6,

NOD, NOD-*scid*, NOD.B10.$H2^b$ and NOD.Igμ^{null} mice. Both male and female mice were used.

Antibodies. Monoclonal antibodies for flow cytometric analysis of the gland infiltrates were purchased from Pharmingen. Specificities include CD3ε, CD4, CD8, CD19, F4/80, and CD11c. The antibodies used in the IgG detection assays include mouse kappa and lambda light chain, and mouse IgG1, IgG2a, IgG2b, and IgG3 (SIGMA).

Tissue Preparation. Submandibular glands, lachrymal glands, spleens, and serum were collected from each animal. Blood was collected from the femoral artery and centrifuged at 1500 x g for 10 minutes at $4^{O}C$ to separate serum for IgG subclass analysis. Submandibular and lachrymal glands and spleen were excised from the animals and placed in PBS. Single cell suspensions were prepared as follows. Splenic leukocytes were obtained by gently pressing the spleen through a wire mesh and washing with PBS.[142] Red blood cells were lysed with 0.84% ammonium chloride. After washing with PBS, remaining leukocytes were divided into the appropriate number of tubes and washed with FACS buffer (PBS with 0.1%NaN_3 (FISHER Scientific) and 0.5% BSA (SIGMA)) prior to antibody staining. For submandibular and lachrymal glands, pooled tissues were minced with scissors prior to enzymatic digestion. Enzymatic digestion was accomplished by suspending tissue samples in a solution of 4mg/ml collagenase type V (SIGMA) and 100U/ml DNase II (SIGMA) for 15 minutes in a $37^{O}C$ shaking water bath. Digested tissues were further dissociated with vigorous pipetting, and transferred to a tube containing a stop solution of ice-cold HBSS containing 2% FBS. Undigested tissues were further treated with 2mg/ml collagenase type V with 100U/ML DNase II in the

37°C shaking water bath for 5 minutes. Digested tissues were separated as above and this last step was repeated until complete digestion of the glands was accomplished.

Leukocytes from the digested lachrymal and submandibular glands were then separated via a 55% Percoll (SIGMA) gradient. Infiltrating immune cells were collected as a pellet and contaminating red blood cells were lysed in 0.84% ammonium chloride. Cells were then divided into the appropriate number of tubes and resuspended in FCS buffer.

Flow Cytometry. Aliquots of all cell populations were resuspended in 100µl of FACS buffer and incubated with fluorescent-labeled antibody at a concentration of 1µg/10^6 cells for 45 minutes at 4°C. In the case of the double-labeled T cells, fractions were first labeled with CD3, and then with either CD4 or CD8. Flow cytometric analyses were performed using the FACScan flow cytometer and LYSIS™ II software.[134] Ten thousand events were counted per sample from a population gated on a window determined as containing the splenic leukocyte population.

RNA Isolation and RT-PCR Detection of Cytokine mRNA. Excised submandibular and lachrymal glands were placed in 10 mM Tris (pH 7.4), homogenized, and placed into storage at –80°C until beginning mRNA isolation. mRNA was isolated using the MicroMACS system (Miltenyi Biotech. Briefly, sample homogenates were incubated with magnetic beads conjugated to poly-T tails for 30 seconds. Homogenate with beads were then run over a column in the presence of a magnet to separate out mRNA. Columns were washed with 4 volumes of wash buffer and then mRNA was eluted off of the column and beads with elution buffer warmed to 65°C. mRNA was quantified for each sample by spectrographic analysis with an A_{260}/A_{280} reading.

RT-PCR amplification of mRNA for cytokine message was performed using the Perkin Elmer RT-PCR kit according to the manufacturer's protocols. Cytokine specific primers were used as defined in Table 1. As a positive control for each cytokine, cultured mouse splenocytes were stimulated with concanavalin A.

IgG Subclass Analysis. Sera from NOD.IL-4$^{-/-}$, NOD.IL-10$^{-/-}$, and NOD.IFN-$\gamma^{-/-}$ mice were collected by femoral bleed while sera from NOD, Balb/c and NOD.Igμ^{null} mice were similarly collected to be used as controls. IgG subclasses were detected by method of sandwich ELISA. Briefly, flat-bottom 96-well ELISA plates were coated with antibodies specific for the kappa and lambda chains of murine IgG overnight at 4^{O}C. Plates were washed with PBS with 0.25% BSA and then incubated with mouse sera samples at dilutions of 1:10,000, 1:25,000, 1:100,000, and 1:250,000 for 4 hours at room temperature. Plates were then washed 3 times in PBS with 0.25% BSA, and wells were exposed to the IgG subclass-specific secondary antibody conjugated to horseradish peroxidase (HRP) for 1 hour at room temperature. Plates were again washed 3 times in PBS with 0.25% BSA and exposed to the enzyme substrate O-phenylenediamine (OPD) for 30 minutes. Plates were then read on a Titer Tek 2.0 ELISA plate reader at 490nm and analyzed using Logit 2.5 software.

Results

Cytokine Analysis. mRNA was purified from the submandibular and lachrymal gland lysates of NOD.IL-4$^{-/-}$ and NOD.IFN-$\gamma^{-/-}$ mice and used in RT-PCR reaction mixtures along with primers to extract message for IL-1β, IL10, IL-12, and IFN-γ, cytokines typically found in association with the onset of SS, as well as G3PDH to serve as a positive control for mRNA isolation. The expression levels of these cytokines were

compared to those previously published in reference to the NOD mouse.[24] As was predicted, there were no significant changes in cytokine expression in either mouse, with the single exception that the NOD.IFN-$\gamma^{-/-}$ mouse did not express any detectable IFN-γ message. IL-1β, IL-10, IL-12 and IFN-γ were all detected at comparable levels by 20 weeks of age in these mice (aside from the above mentioned exception) (Fig. 4-1*a*). Of note, several other inflammatory cytokines including IL-6, and TNF-α were assayed for and detected in the NOD.IFN-γ background, on the grounds of exploring whether these mice expressed the typical pro-inflammatory cytokine profiles despite lacking the prototypical T_H1–type cytokine, IFN-γ. Interestingly, IFN-γ was also detected in the exocrine gland lysates of the NOD-*scid* mouse (Fig 4-1*b*), suggesting that the effects of this cytokine may precede and potentiate autoimmune lymphocytic infiltration of the exocrine glands.

Flow Cytometry. Since the NOD.IFN-$\gamma^{-/-}$ mouse does not develop focal infiltrates in the salivary tissues until much later in life, we concentrated on the NOD.IL-$4^{-/-}$ mouse in the flow cytometric analysis of the infiltrating lymphocytes. As a control tissue for the flow cytometric analysis, spleens were also harvested and evaluated in terms of leukocyte populations. Based on histological visualization, the IL-4-deficient NOD congenic mouse develops a more pronounced focal inflammation of the salivary and lachrymal glands and the glands show signs of acute inflammation slightly earlier than the NOD parental strain (see chapter 3). These findings were consistent with initial observations in the flow cytometric analysis in that there were significantly more lymphocytes per 10,000 cells counted (Table 4-2*a*.). Aside from the apparent increase in leukocytic infiltration, there were no significant shifts in the subpopulation composition of these cells in the

Table 4-1. Murine Cytokine Primers

Cytokine	5' Primer	3' Primer	Amplicon Size
IL-1β	5'-TGGCAACTGTTCCTGAACTCAACT	5'-CAGGACAGGTATAGATTCTTTCCTTT	536bp
IL-2	5'-ATGTACAGCAATGAGCTCGCATC	5'-GGCTTGTTGAGATGATGCTTTGACA	502bp
Il-6	5'-ATGAAGTTCCTCTCTGCAAGAGACT	5'-CACTAGGGTTTGCCGAGTAGATCTC	638bp
IL-10	5'-GCAGGGGCCAGTACAGCCGGGAA	5'-GCTTTCATTTTGATCATCATGT	479bp
IFN-γ	5'-TGAACGACAGAAAGCATGATCCGC 5'	5'-CGACTCCTTTTCCGCTTCCTGAG	460bp
TNF-α	5'ATGAGCACAGAAAGCATGATCCGC	5'-CCAAAGTAGACCTGCCCGGACTC	692bp
iNOS	5'-CCCTTCCGAAGTTTCTGGCAGCAGC	5'-GGTGTCAGAGCCTCGTGGCTTTGG	497bp
G3PDH	5'-TGAAGGTCGGTGTGAACGGATTTGGC	5'-CATGTAGGCCATGAGGTCCACCAC	983bp

submandibular glands. The ratio of B-cells to $CD4^+$ T-cells conformed to the previously established ratio[24] of approximately 1:2 (see Table 4-2*b*.). However, the lachrymal glands of the NOD.IL-$4^{-/-}$ mouse showed a slight skewing of this B:T-cell ratio in favor of the B cell and, furthermore, these glands were much more heavily infiltrated than either the salivary glands of these animals or the lachrymal glands of the NOD parental control strain.

IgG Subclass Analysis. As neither the cytokine expression profiles nor the breakdown of the infiltrating cell populations indicated any significant trends that might begin to explain the normal salivary function observed in the IL-4- or IFN-γ–deficient NOD mouse models, we next turned to the characterization of the serum immunoglobulin to look for alterations in the disease pathogenesis that might correlate to the maintenance of normal secretory capacity. Evaluation of the presence of specific IgG subclasses revealed an interesting trend in subclass expression. NOD mice showed similar IgG subclass distributions to BALB/c control mice (Fig. 4-2). The NOD.IL-$4^{-/-}$ produced virtually no IgG1, as would be expected due to the deficiency, but this was significantly less than amounts found in the NOD (Fig. 4-2*a*). The IFN-γ- and IL-10-deficient NOD strains each produced significantly greater IgG1 than the NOD. Notably, the NOD.IFN-$\gamma^{-/-}$ exhibits normal salivary capabilities, while the NOD.IL-$10^{-/-}$ mouse develops severe focal inflammation of the exocrine glands and experiences a drop in secretory flow rates. All other IgG subclasses are expressed at relatively comparable levels in these mice (data not shown), although the IFN-γ-deficient mice do not produce as much IgG3 as the

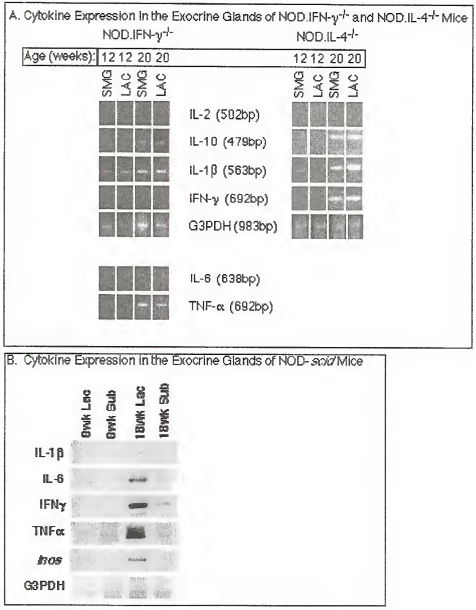


Figure 4-1. Analysis of cytokine mRNA expression in the submandibular and lachrymal glands. Glandular mRNA was isolated and amplified by RT-PCR. In panel A, cytokine specific primers were used to detect IL-1β, IL-2, IL-10 and IFN-γ in both NOD.IL-4$^{-/-}$ and NOD.IFN-γ$^{-/-}$ mice. Additionally, NOD.IFN-γ$^{-/-}$ glands were probed for IL-6, iNOS and TNF-α expression to evaluate the inflammatory response in these mice in the absence of IFN-γ. In panel B, mRNA was probed using primers for inflammatory cytokines in the exocrine glands of NOD-*scid* mouse. G3PDH was detected as an internal control for standardization of samples.

Table 4-2a. Flow cytometric analysis of the infiltration leukocyte population in the salivary and lachrymal glands of NOD.IL-4$^{-/-}$ *vs* NOD.B10.H2^{b} mice.

	NOD.B10.*H2*^{b}			NOD.IL-4$^{-/-}$		
CD Marker	**Submand.**	**Lachrymal**	**Spleen**	**Submand.**	**Lachrymal**	**Spleen**
12 Week Animals						
CD3^{+}	0.2%	3.5%	40.15%	1.4%	23.7%	41.5%
CD3^{+}/CD4^{+}	0.2%	1.4%	29.9%	0.8%	13.5%	28.6%
CD3^{+}/CD8^{+}	0	0.4%	3.3%	0.3%	4.5%	7.7%
CD19^{+}	0.1%	1.5%	47.3%	0.3%	42.6%	40.5%
16 Week Animals						
CD3^{+}	4.1%	15.8%	60.0%	21.6%	31.3%	66.1%
CD3^{+}/CD4^{+}	0.7%	2.2%	35.2%	13.1%	16.9%	42.0%
CD3^{+}/CD8^{+}	0.6%	1.8%	14.3%	4.6%	6.7%	12.3%
CD19^{+}	0.3%	2.0%	32.5%	6.6%	50.0%	36.6%

Table 4-2b. Ratio of B cells : T cells in theExocrine Glands

	Submandibular	**Lachrymal**
NOD.B10.*H2*^{b}		
12 week	1 : 2	1 : 1
16 week	1 : 3	1 : 1
NOD.IL-4$^{-/-}$		
12 week	1 : 2	3 : 1
16 week	1 : 3	3 : 1

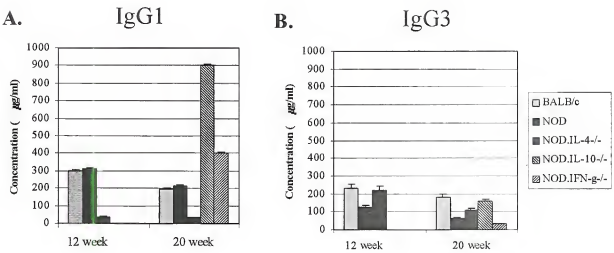


Figure 4-2. Serum IgG subclass analysis. Serum samples from 12 and 20 week old BALB/c, NOD, and NOD.IL-4$^{-/-}$ as well as from 20 week old NOD.IL-10$^{-/-}$ and NOD.IFN-$\gamma^{-/-}$ mice were diluted 1:25,000, 1:100,000, 1:250,000 and 1:500000 and evaluated for the presence of the 4 IgG subclasses by ELISA. Panel A represents IgG1, and panel B represents IgG3.

BALB/c control and the IL-4-deficient NOD produces slightly higher amounts of this subclass than any other strain tested (Fig. 4-2*b*).

Discussion

The immune response has been well documented as driving the loss of secretory function in the NOD mouse model of SS and has helped to provide ample evidence that this same mechanism exists in the human disease. However, the specific mechanism by which the immune system generates this effect remains to be fully elucidated. From prior studies, in the NOD background, the B cell has emerged as a critical effector cell, and passive transfer studies of human SS patient IgG into NOD.Igμ^{null} mice have further indicated that antibodies produced by the autoreactive B cells are the true culprits driving the secretory hypofunction[120]. Interestingly, studies in the IL-4 and IFN-γ cytokine-deficient NOD mice exploring the effects of immune deviation on the pathogenesis of autoimmune exocrinopathy suggest that both of these cytokines play critical roles in the development of exocrine dryness. The work presented here further probes into the mechanism by which the autoimmune attack on the salivary glands results in the generation of the autoantibodies that are responsible for the loss of secretory capacity.

The analysis of cytokine mRNA expression in these congenic NOD strains suggests that the underlying cytokine expression profiles remain consistent with those found in the NOD parental strain, with the noted exception of IFN-γ lacking in the NOD.IFN-$\gamma^{-/-}$. Even in the absence of this cytokine, though, message can still be detected for other pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α. The cytokine expression in the NOD.IL-$4^{-/-}$ mouse is consistent with predictions, based on the histological assessment of

increased inflammation and focal infiltration and the lack of IL-4 expression in the autoimmune lesions.

However, recent studies have detected message for the proinflammatory cytokines reportedly involved in SS pathogenesis expressed in the salivary glands of healthy individuals as well as in the salivary glands of non-exocrinopathic mice.[50,51] Thus, the detection of mRNA for these cytokines might not be as impressive as the detection of their activity. Regardless, these data suggest that the NOD.IL-4$^{-/-}$, although not necessarily the NOD.IFN-$\gamma^{-/-}$ mouse appears to maintain cytokine message at similar levels as the NOD model, an animal that shows a slight increase in message levels over control non-autoimmune strains.

Similar to the cytokine analysis, the characterization of the infiltrating leukocyte populations in the salivary and lachrymal glands indicate very little difference between the IL-4-deficient NOD animal and the NOD parental strain. Flow cytometry confirmed previous histological observations of increased glandular infiltration, especially in the lachrymal glands, at earlier time points, but the ratios of cell populations tended to remain consistent. One potentially interesting difference observed was the increase in infiltrating B-cells both in shear numbers and in relation to other cell subpopulations in the lachrymal glands but not the salivary glands of the NOD.IL-4$^{-/-}$ mouse as compared to the age-matched NOD animal. This supports earlier evidence from the initial characterization of the NOD.IFN-$\gamma^{-/-}$ mouse that the salivary and lachrymal pathologies are potentially independent autoimmune manifestations. These infiltrating B cells will need to be further characterized in the future to identify any subtle changes in cell

phenotype that may contribute to the altered pathogenesis and maintenance of exocrine gland function.

In contrast to the cytokine and flow cytometry data, the analysis of IgG subclass distributions revealed a couple of interesting trends. While serum levels of IgG2a, IgG2b, and IgG3 tended to remain consistent between the various congenic strains tested, IgG1 seemed to be the most interesting of the four subclasses. First of all, the absence of IL-4 produces a dramatic decrease in IgG1 as compared to the NOD model of the disease as expected.[143] This suggests that, in the presence of severe inflammation and focal infiltration as seen in the NOD.IL-$4^{-/-}$ mouse, the absence of any detectable IgG1 response could be a significant difference that accounts for the normal glandular function in these animals.

However, the IFN-γ-deficient mouse, which also maintains normal secretory capacity, generates higher amounts of IgG1 than the NOD parental strain. This could potentially be explained, in part, by the argument that the immune response in these animals is skewed towards a T_H2-type humoral response, which would be associated with higher levels of IgG1. Histological examination of the exocrine tissues of these mice revealed that the salivary glands remained largely free of infiltrates, while the lachrymal glands developed overwhelming leukocytic infiltrates by 20 weeks of age. Thus, the increase in IgG1 in these animals may also reflect the ongoing immunological infiltration and activation in the lachrymal glands, resulting in production of this particular Ig isotype, although, not the specific autoantibodies responsible for the loss of glandular function. The fact that these animals do not develop appreciable inflammation in the salivary glands, suggests that IFN-γ is critical for the development of the salivary aspects of this

disease and that the decrease in exocrine function is dependent on the development of salivary gland autoreactivity. If the elevated serum IgG1 levels were truly a repercussion of the ongoing lachrymal pathology, this would have tremendous implications with regard to the pathogenesis of autoimmune exocrinopathy in the NOD mouse model.

In essence, this work provides several novel and interesting insights into the immunological mechanisms involved in the pathogenesis of autoimmune exocrinopathy in the NOD mouse. First, the NOD mouse may be, in reality, an animal model for the manifestation of two distinct, yet interrelated, diseases in oral and ocular autoimmune exocrinopathy. And second, the mechanism for immune modulation of exocrine function may be dependent on the effective generation of IgG1, and furthermore, this is reliant not only on the appropriate cytokine milieu (*ie.* the presence of IL-4), but also on a specific tissue or environment.

CHAPTER 5
INTRACELLULAR SIGNALING INDUCED BY SJÖGREN'S ANTIBODIES

Introduction

Salivary and lachrymal exocrine gland secretory responses are regulated by the enervating sympathetic and parasympathetic nerves and the autonomic neurotransmitters they produce.[117,118] In these glands, the acinar cell population serves as the primary source for many of the salivary and lachrymal proteins that comprise saliva and tears. Additionally, these cells are instrumental in the generation of the fluid phase of saliva. Signals received at the basal surface of these cells stimulate the trafficking of proteins, electrolytes and water through the cell to the apical surface where these components are released in the exocrine ductal system. The secretion of the proteinaceous phase of saliva is stimulated primarily by the β-adrenergic receptor, while the fluid phase of saliva is produced primarily in response to signaling through the muscarinic receptor,[117,118] although several other neuropeptides have also been shown to contribute to the stimulation of salivary secretion, including Substance P and vasoactive intestinal peptide (VIP).[144,145,146]

Several of these receptors, mainly the muscarinic receptor, have recently come under intense scrutiny with respect to their potential involvement in the human autoimmune

disease, Sjogren's syndrome (SS). SS patients exhibit an immune-mediated loss of secretory function in their salivary and lachrymal glands. The disorder is characterized by focal lymphocytic infiltration of the exocrine glands corresponding to the production of a number of identified autoantibodies and the destruction of the glandular acinar cell populations.[147] Recent evidence has begun to suggest that the loss of glandular function, however, may be mediated by the autoantibodies and not simply the destruction of the secretory acinar cell population by the focal inflammatory response.[127] Furthermore, anti-muscarinic antibodies, directed against the M_3 subtype specifically, have been detected in the serum of SS patients,[70] suggestive of a more intricate mechanism by which SS autoantibodies drive secretory hypofunction.

If the anti-muscarinic (M_3R) antibodies are responsible for driving the loss of secretory function, they could, in theory, be acting through one of several possible mechanisms. They could be interfering with receptor signaling capacity by blocking the initiation of the signal by either blocking neurotransmitter binding, or inhibiting receptor conformational shifts essential for transmembrane signal transduction. Alternately, these antibodies could induce receptor internalization by occupying and potentially stimulating or overstimulating receptor signaling.[148,149] Furthermore, if these antibodies are able to generate an intracellular signal, there is the potential that they are initiating a signal cascade that results in the activation of programmed cell death (PCD), thus contributing to the elevated levels of apoptosis detected in the exocrine glands of SS patients and in animal models of the disease.[32,94,96] Thus, there is ample reason to focus on the anti-muscarinic antibody response as a potential effector molecule in the SS pathology.

To date, muscarinic signaling has been fairly well characterized. The receptor family includes 5 subtypes, M_1 through M_5, and each member contains 7 transmembrane domains.[150] The M_3 subtype is predominantly responsible for fluid secretion in the salivary and lachrymal glands[117] and interacts with $G_{q/11}$ subtypes of G-proteins to activate phospholipase C.[151] This signal cascade can be traced through the activation of protein kinase C (PKC) and the mitogen activated protein kinases (MAPK). In the case of the MAPK, extracellular signal regulated kinsases (ERK) are activated more rapidly but are more transiently active than the stress-activated protein kinases (SAPK) which appear to undergo a delayed but more sustained activation.[152] Additionally, ERK activation seems to be dependent on PKC activity but not Ca^{2+} mobilization, whereas SAPK activation appears to be PKC independent but associated with increases in cytosolic Ca^{2+} ions.[151] Stimulation of the muscarinic receptors is also known to induce the translocation of aquaporins (AQP) to the apical plasma membrane in a Ca^{2+}-dependent fashion in rat parotid gland ductal cells,[153] contributing to enhanced fluid secretion.

The purpose of these studies is to further elucidate the mechanism by which SS autoantibodies are driving the loss of secretory function and determine the specific role of the anti-muscarinic antibodies in terms of exocrine dysfunction. Using specific anti-muscarinic antibodies, as well as IgG purified from the serum of SS patients or of healthy donors, we looked for the induction of intracellular signaling activity consistent with muscarinic receptor stimulation, including inositol phosphate (IP) metabolism leading to CA^{2+} mobilization, AQP translocation, and MAPK phosphorylation. Based on evidence connecting SAPK activation to the induction of apoptosis,[154,155] we also explored the

ability of these antibodies to induce caspase-3 activation. Thus, we sought to evaluate the possibilities that the SS autoantibodies and, more specifically, the anti-muscarinic antibody, could potentially be regulating secretory function by either interfering with signaling leading to the loss of AQP activation, or by targeting the secretory cell populations for apoptosis. In this study, we found that the submandibular acinar cell line SMG C6, cultured with patient sera, showed signs of elevated cytosolic Ca^{2+}. Culturing these cells in the presence of either SS patient serum IgG, or monoclonal antibodies directed against the M_3R, compared to appropriate controls, produced no appreciable caspase-3 activity. Furthermore, there was no significant MAPK activity associated with cell cultures treated with these antibodies. Chronic infusion with monoclonal antibodies directed against the M_3R resulted in decreased AQP translocation in primary acinar cells isolated challenged with a muscarinic agonist. Thus, it would appear that SS serum autoantibodies direct the loss of secretory function mainly by inhibiting water channel translocation.

Materials & Methods

Materials. BALB/c mice were bred and maintained under specific pathogen-free conditions in the mouse facility at the University of Florida. SMG C6 cells were a kind gift from Dr. Dave Quissell and were maintained in the laboratory at $37^{O}C$ in a CO_2 incubator. Anti-M_3R and isotype control IgM antibodies were produced in conjunction with the University of Florida Hybridoma Core Facility.[156] The anti-La/SS/B antibodies were a kind gift from Dr. Bachmann. AQP antibodies were a gift from Dr. Bruce Baum at the NIH, SS patient antibodies were generously donated by Dr. Roland Jonsson

(Broegelmann Research Laboratory, Bergen NO) and by Dr. Marjan Versnel (Erasmus University, Rotterdam, ND).

IgG preparation and injection into mice. Sera were isolated from whole blood collected from patients diagnosed with primary Sjögren's syndrome (SS) meeting the following diagnostic criteria: 1) the loss of saliva and tear production, 2) positive Rose Bengal staining, 3) lymphocytic foci in minor labial gland biopsies, and 4) the presence of antinuclear SS-A/Ro and SS-B/La autoantibodies in serological evaluations.[100,101] At the time of serum collection, the patients were determined to be free of other complicating autoimmune diseases. IgG fractions were isolated by standard protein A-agarose, dialyzed to remove excess salts, and lyophilized to concentrate the antibodies. The purity of the IgG fractions was assessed at >90% by SDS/PAGE.

AQP Activation Assay. NOD-*scid* or C57BL/6-*scid* mice were injected once per day for three days with either anti-M_3R or control IgM antibody. Submandibular glands were excised from mice after the three day treatment and freed of connective tissues to form a single cell suspension. Briefly, pooled tissues from each treatment group (n = 5) were minced with scissors prior to enzymatic digestion. Enzymatic digestion was accomplished by suspending tissue samples in a solution of 4mg/ml collagenase type V and 100U/ml DNase II for 15 minutes in a 37^{O}C shaking water bath. Digested tissues were further dissociated with vigorous pipetting, and transferred to a tube containing a stop solution of ice-cold HBSS containing 2% FBS. Undigested tissues were further treated with 2mg/ml collagenase type V with 100U/ML DNase II in the 37^{O}C shaking water bath for 5 minutes. Digested tissues were separated as above and this last step was repeated until complete digestion of the glands was accomplished. Cells were then

cultured for 1 hour in RPMI-1640 complete media with 5% FBS. Cultures were then supplemented with either 1μM carbachol or an equal volume of saline. Five minutes after the addition of carbachol or saline, cells were lysed by rapid freeze-thaw and lysates were separated into cytosolic and membrane fractions via centrifugation at 15,000x g for 20 minutes at 4^{o}C following a 500xg spin to remove the nuclei and unlysed cells. AQP translocation was evaluated by Western blot analysis of the cellular fractions using antibodies specific for AQP.

Western blot analysis was performed by first separating cell lysates (25 μg/well) on 12% SDS-polyacrylamide gels.[66,69] The proteins were transferred to PVDF membranes, blocked in TBS containing 3% BSA, and incubated with a rabbit anti-mouse AQP resuspended in TBS containing 3% nonfat milk (NFM). Membranes were washed three times in wash buffer (TBS with 0.1% Tween-20), and then incubated with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody. Bands were detected by exposing the membranes to ECL chemiluminescent substrate (Amersham) for 1 minute and then exposed to film for 30 seconds. The AQP was detected as a band corresponding to 27 kDa in molecular weight.

Caspase-3 Activity Assay. SMG C6 cells grown to confluence and then cultured in the presence of SS patient IgG, control human IgG, or in the presence of the monoclonal antibodies, anti-La/SS-B, anti-M_3R, and the M_3R isotype control for 24 hours. Caspase-3 activity was measured using the FluorAce Apopain Detection kit (BioRad). After 24 hours, cells were prepared according to manufacturers protocols with adjustments to the final reaction volumes to allow for analysis on a microtiter plate reader. In brief, cells were harvested in caspase-3 lysis buffer (10mM HEPES, 0.1% CHAPS, 1mM

dithiolthreitol (DTT), 1μM PMSF, 10μg/ml aprotinin, 10μg/ml pepstatin, 5μg/ml leupeptin) and lysed by repeated freeze-thaw and then by centrifugation at 15,000x g for 15 minutes at 4°C. Lysates were then aliquoted into wells of a 96-well ELISA plate in addition to reaction buffer and the caspase-3 substrate AFC-DEVD-CHO. Enzyme activity was measured as the increase in fluorescence over time on a TECAN Dual Wavelength Fluorimeter and analyzed using Microsoft Excel 5.0 software.

MAPK Activation Assay. SMG C6 cells grown to confluence and then cultured in the presence of SS patient IgG, control human IgG, or in the presence of the monoclonal antibodies, anti-La/SS-B, anti-M_3R, and the M_3R isotype control for 3 hours. The SAPK and ERK families of MAPK were evaluated for their roles in intracellular signaling by Western blot analysis. In brief, antibodies directed against SAPK (New England BioLabs, Inc.) were used to detect protein expression levels, while antibodies directed against the phosphorylated versions of SAPK and ERK (New England BioLabs, Inc.) were used to detect specifically activated forms of these signaling proteins.

Cell lysates were prepared according to the suggested protocol in a lysis/loading buffer (62.5mM Tris-HCl (pH 6.8), 2% w/v SDS, 10% glycerol, 50mM DTT, 0.1%w/v bromphenol blue) and 20 μl of sample were run on a 10% SDS-PAGE gel, and transferred to PVDF membranes. Membrane blots were first blocked in 1x TBS, 0.1% Tween-20 with 5% w/v nonfat milk for 2 hours at room temperature, then incubated overnight at 4°C in primary antibody (1:1000 dilution) in 1x TBS, 0.1% Tween-20 with 5% bovine serum albumin. Blots were then washed in 1x TBS, 0.1% Tween-20 3 times for 5 minutes, and incubated for 1 hour at room temperature in secondary antibody (HRP-conjugated goat-α-rabbit IgG, 1:2000) resuspended in blocking buffer. MAPK detection

was visualized by enzyme-linked chemiluminescence (ECL) (Amersham) and exposed to film for 10 seconds. The 2 ERK isoforms are detected at 42 and 44 kDa while the α- and β- isoforms of SAPK are detected at 46 and 54 kDa (the γ-isoform is not detected by these antibodies).

Inositol Phosphate Metabolism Assay. IP, a second messenger, was evaluated for its activity in the intracellular signaling mechanism of the M_3R based on a protocol defined by Muhoczy-Dominiak and Garg.[157] The assay was performed on both primary isolated cells from murine submandibular glands and a rat acinar cell line grown in culture. Salivary cells for the cell isolates were prepared from BALB/c mice and resuspended in Kreb's-Hensleit buffer (KHB). IgG isolated from SS patients or healthy control individuals (10^{-6}M, 300μl) was added to each tube and the suspension was incubated for 1 hour at 37°C. One hundred μl of 5μM carbachol solution was added to each tube followed by 20μl of 1μCi of myo-[^{3}H]inositol. The tubes were gassed with 95% O_2 / 5% CO_2 and incubated for 30 minutes at 37°C. One mL of (1:2) chloroform/methanol was added along with 0.35ml of chloroform and 0.35ml of water with 5ml of HCl. The solution was vortexed and centrifuged. Inositol phosphates were purified from 1.2ml of the supernatant through column chromatography. Elution of inositol phosphates was achieved through a 5ml gradient of ammonium formate/formic acid. Each sample was then solubilized by 10ml of counting cocktail and measured in a scintillation counter.

Salivary cells from cultured cell lines were prepared with 50,000 cells per well. SS or healthy donor IgG was added and incubated exactly as above. The supernatant was discarded and 230μl of 1μM carbachol or KHB was added to each well, along with 20μl

of 1μCi of myo-[^{3}H]inositol. This was incubated in a CO_2 incubator for 30 minutes at 37^{o}C. The supernate was collected into a glass culture tube and the wells were washed with 250μl of KHB to bring the total sample volume up to 500μl. Aside from harvesting the cells and preparation, the experimental steps in this procedure are identical to those detailed above.

Results

To begin to determine the influence of SS autoantibodies on M_3R signaling, we first looked at the ability of patient serum IgG to alter normal M_3R signaling. Signaling through the M_3R leads to a complex sequence of events encompassing the activation of several pathways. The initial events in the M_3R signal cascade involve the Gq/11-mediated activation of phospholipase C (PLC). This leads to rapid generation of inositol-1,4,5-triphosphate (IP_3) and Ca^{2+} mobilization, one of the critical second messengers in the M_3R signaling cascade. Figure 5-1 depicts the modulation of IP metabolism through M_3R signal transduction following preincubation with SS autoantibodies. IP metabolism was measured as the production of IP_3 and the intermediary products leading to IP_3 formation from precursor insoitol phosphate via phospholipase C-γ activity. SS patient serum IgG, but not IgG purified from healthy donor serum contains antibodies that can inhibit normal IP_3 metabolism resulting from the stimulation of the muscarinic receptor with the specific agonist, carbachol. In data not presented, several patient IgG samples were shown to have the capacity to induce low levels of M_3R signaling (as measured by IP metabolism) although these antibodies still blocked receptor function prior to the presence of 1μM carbachol.

SS and murine autoimmune exocrinopathy both present with elevated apoptotic activity in the salivary and lachrymal glands.[93] Therefore, we chose to explore whether the loss of gland function could result from the chronic or persistent signaling through the muscarinic receptor, leading to the induction of caspase-3 and PCD in the SMG C6 cell line.

Caspase-3 activity was assessed by incubating 200,000 SMG C6 cells, grown to confluence, in the presence of $5x10^{-8}$M, 10^{-7}M, or $2x10^{-7}$M antibody and additionally in the presence or absence of 1μM atropine for 24 hours. The concentrations used were determined on the basis of previous studies indicating that purified SS serum IgG at a concentration of 10^{-7}M could both inhibit quinuclidinyl benzilate (QNB) binding to muscarinic receptors by 50% and induce salivary dryness when passively transferred into NOD.Igμ^{null} mice.[127] Cells cultured with M_3R antibodies up to a concentration as high as $2x10^{-7}$M showed no detectable caspase-3 activity above the control cultures incubated with identical concentrations of the isotype control (Fig. 5-2a). Similar results were found when measuring the ability of IgG from SS patient sera as compared to IgG from the sera of healthy controls (Fig. 5-2b). Very little caspase-3 activity was observed above levels seen in control cultures at antibody concentrations of 10^{-7}M.

Although the antibodies did not seem to be able to directly induce apoptosis in the cell line, they could still predispose target cells to facilitate the induction of PCD by other factors. Therefore, we decided to test whether this signal cascade might involve the MAPK's. SAPK activation has been linked in some cases to the activation of PCD, whereas ERK seems to be associated more closely with proliferative events, although the progression to cellular apoptosis or proliferation are probably more a reflection of the

relative activities of these kinases.[158] Therefore, we investigated the activation states of both the ERK and SAPK families.

SMG C6 cells were cultured under the same conditions as with the caspase-3 experiments; however, the incubation period was reduced to 3 hours based on initial time course experiments that determined optimal durations for MAPK activity (data not shown). The activation of the SAPK and ERK proteins was detected via Western blot using antibodies recognizing the phosphorylated (activated) enzyme. Similar to the caspase-3 results, very little SAPK or ERK activation was detected above levels seen in any samples from cells exposed to 10^{-7}M concentrations of antibody (Fig. 5-3 *A* and *B*). However, SAPK appeared to undergo limited phosphorylation in a concentration dependent manner at higher concentrations (Fig. 5-4). Atropine had no effect on this activation and the independence of this signal from muscarinic stimulation was supported by the lack of phosphorylation of SAPK in response to increasing concentrations of M_3R monoclonal exposure (Fig. 5-4). Additionally, the expression of the SAPK protein was significantly increased in cells treated with SS patient IgG and the presence of 1μM atropine inhibited this upregulation (data not shown). Thus, MAPK activity detected in association with SS patient serum IgG appears to be independent of M_3R stimulation, but seems to result, rather, from the specific activity of an unidentified autoantibody.

In the exocrine glands, Ca^{2+} mobilization leads to several important events that translate to the movement of water into the exocrine ductal system. The secretion of water relies on the channeling of ions to establish a chemiosmotic gradient that water will follow through the acinar cells into the secretory ducts.[159] Additionally, this transfer of water is facilitated by AQP's, a family of proteins that channel water through the plasma

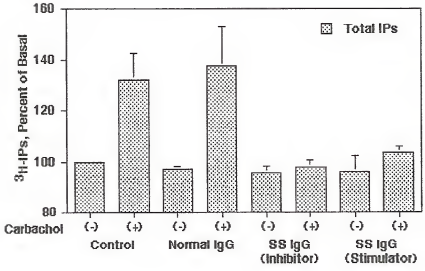
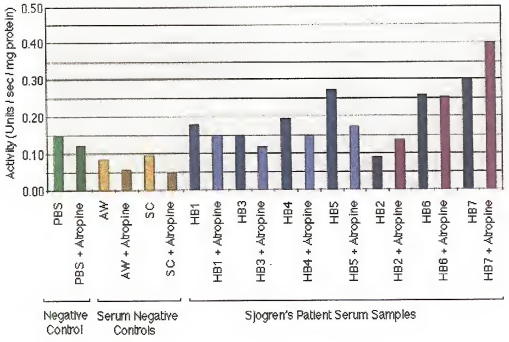


Figure 5-1. Inhibition of IP metabolism in response to muscarinic agonist by SS autoantibodies. SMG C6 cells were incubated in the absence (-) or presence (+) of carbachol with culture media (Control) or IgG purified from the sera of healthy individuals (Normal IgG) or SS patient sera (SS IgG). Inhibitor and stimulator labels refer to the effects on stimulated salivary secretion when passively transferred into recipient mice.

A.



B.

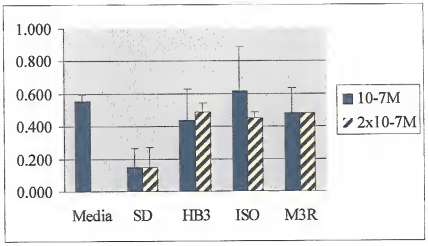


Figure 5-2. Caspase-3 activity measured in SMG C6 cells incubated 24 hours in the presence of serum antibodies. In figure 5-3*a*, caspase-3 activity was measured in the cells in response to treatment with 10^{-7}M concentrations of antibodies. In figure 5-3*b*, titration studies were performed to confirm antibody-mediated caspase activity. AW, SC and SD represent cells treated with serum IgG samples from healthy donors, HB1-7 are cells treated with SS patient IgG samples, M3R represents cells treated with the monoclonal M3R antibody and ISO refers to cells treated with the istotype control. Media or PBS serve as negative controls.

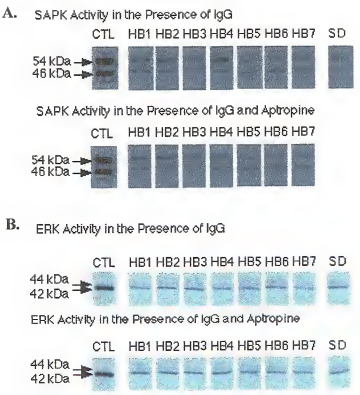


Figure 5-3. Phosphorylation of MAPK in response to SS Autoantibodies. SMG C6 cells were incubated for 3 hours in the presence of antibodies purified from the serum of healthy donors (SD) or SS patients (HB1-7), and in the presence or absence of atropine. Activated (phosphorylated) SAPK (A) and ERK (B) were detected via Western blot.

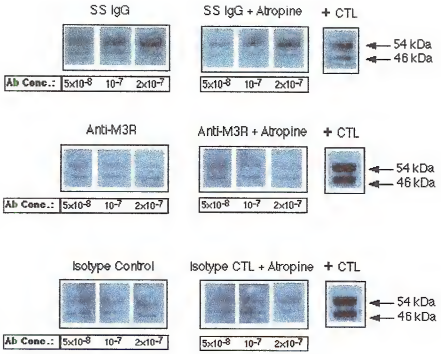


Figure 5-4. Phosphorylation of SAPK in response to varying concentrations antibodies. SMG C6 cells were incubated for 3 hours in the presence of antibodies purified from the serum of SS patients (SSIgG) or in the presence of monoclonal antibodies directed against the M_3R or an isotype control. Activated (phosphorylated) SAPK was detected via Western blot.

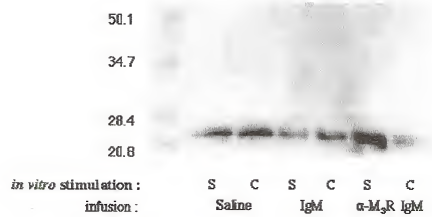


Figure 5-5. Effects of monoclonal M_3R passively transferred *in vivo* into NOD-*scid* mice on AQP translocation. Translocation of AQP was determined by Western blot analysis of membrane cell fractions. Lanes 2 and 3 represent the membrane fractions of saline or carbachol stimulated cells from animals treated with saline; lanes 4 and 5 are similarly treated fractions from animals treated with the isotype control antibody, and lanes 6 and 7 are fractions from animals injected with the the M_3R antibody. Lane 1 contains the standard. AQP shows up as a band at approximately 27 kDa.

membrane. The muscarinic receptor has been shown to induce both the ion flux and the translocation of AQP to the apical surface membrane in a Ca^{2+}-dependent manner.[160] Therefore, we tested the alternate hypothesis that the M_3R autoantibodies could be interfering with cellular secretory function by inhibiting AQP translocation to the plasma membrane.

NOD-*scid* mice were treated for three consecutive days with intraperitoneal injections of the anti-M_3R monoclonal antibody. Following treatment animals were sacrificed and submandibular glands were excised, dissociated into single cell suspensions and briefly exposed to carbachol after a 1 hour acclimatization period in culture media. Cellular response to carbachol was stopped by snap-freezing cells and crude membrane preparations, enriched for plasma membrane, were isolated by differential centrifugation. Figure 5-5 depicts preliminary evidence that the M_3R antibodies, previously shown to be capable of inducing salivary secretory suppression in mice when injected intraperitoneally,[156] disrupted the translocation of AQP to the plasma membrane. Thus, the mechanism by which the anti-M_3R monoclonal antibodies, and presumably SS autoantibodies, drive the loss of glandular function may rely, at least in part, on the ability of these antibodies to dampen the signal that evolves into the facilitation of water translocation via AQP activation.

Discussion

The results of these experiments begin to bring into focus our perspective on a potential underlying mechanism by which the autoantibodies found in SS patient serum bring about the functional suppression of the salivary and lachrymal glands. Several important studies over the last few years have provided critical evidence suggesting M_3R

interaction and signaling in response to the presence of SS autoantibodies.[127,161,162] Most recently, Waterman, *et al.* (2000) demonstrated that SS serum IgG inhibits carbachol-induced bladder contrations[162] However, none of these prior studies ever definitively demonstrated a direct connection between the interaction of the autoantibodies with their target molecules and the suppression of secretory capacity. These data indicate that the SS patient serum IgG and the anti-M_3R antibody act to inhibit cellular secretory function in a manner independent of caspase activity and MAPK activation. Furthermore, this set of experiments presents preliminary data to suggest that SS autoantibodies regulate secretory function by interfering with the signaling pathway that results in the translocation of AQP to the apical cell surface.

SS patient IgG, unlike monoclonal antibodies directed against the M_3R, were able to induce increased caspase-3 activity in a concentration-dependent manner in the SMG C6 cell line, but this was only seen at extremely high concentrations of antibody, 3 times the concentration demonstrated to effect secretory hypofunction when injected intraperitoneally into mice[127] (data not shown). Considering this proteolytic activity could not be detected at physiologically active concentrations, there is good reason to question the relevance of this activity with respect to the disease state in the exocrine glands of SS patients. Similar to the caspase data, the activation of the MAPK pathway, and more specifically the SAPK pathway does not appear to occur at physiologically relevant antibody concentrations. SS patient IgG was able to induce a slight increase in SAPK activity but only at excessively high concentrations and this was not altered by the presence of atropine. Consistent with this observation, the M_3R monoclonal antibody was not able to induce any detectable phosphorylation of the SAPK protein. Together,

these data indicate that the antibodies directed against M_3R in SS patient serum do not appear to be capable of disrupting glandular function by inducing apoptosis through a caspase-3- and SAPK-mediated signal pattern. Finally, any observed caspase or SAPK activity associated with SS patient sera would appear to be occurring independently of M_3R interactions.

The reduction in IP metabolism (IP, IP_2, and IP_3) associated with the SS patient serum IgG provides support for the conclusion that these antibodies can functionally disrupt cellular activity by interfering with muscarinic signaling. Hence, these data are consistent with the prior studies suggesting that autoantibodies function as receptor antagonists.[127,161,162] *In vivo*, this appears to lead to a reduction in AQP translocation. Thus, these experiments depict a consistent effect that can be traced through multiple steps in the secretory signaling process. While suggestive of this mechanism, the AQP translocation study is compromised in its reliability due to the lack of specificity in the cell membrane preparations. While the crude membrane preparation strategy employed may enrich for the larger membrane sheets such as the plasma membrane, it does not exclusively isolate this fraction. The data from this study will therefore need to be confirmed by repeating the experiment and isolating purified plasma membrane fractions using established sucrose gradient centrifugation protocols and including pertinent controls showing the consistent presence of a plasma membrane-specific protein marker. Despite these technical shortcomings, the detection of reduced levels of AQP in the crude membrane preparations serves as encouraging preliminary evidence to support the hypothesis that the suppression of glandular secretory function results from the inhibition of AQP translocation to the cell surface.

Nonetheless, the results presented in these studies allow us to propose that the loss of secretory function in SS patients and in the NOD mouse model of SS appears to occur independently of the induction of apoptosis. Instead, secretory inhibition appears to be enforced at least in part by the antibodies through the antagonism of the M_3R signal transduction pathway leading to AQP activation and translocation.

CHAPTER 6
CONCLUSION

The central objective of these experiments was to evaluate the roles of autoantibodies and apoptosis in SS and, furthermore, to determine any relation between these two pathological aspects of the disease. Secretory dysfunction is a hallmark manifestation in SS, and yet the mechanism by which this occurs is still not clear. The predominance of $CD4^+$ T cells in the focal glandular infiltrates supports theories that functional deficiencies are the result of the inflammatory response. Further histological analysis of patient salivary biopsies provides evidence for a dramatic loss of the acinar cell population and additionally indicates an increase in apoptotic activity in the exocrine tissues of SS patients, suggesting that the loss of function could result from the apoptotic elimination of the secretory cell population. Additionally, the disease is associated with serum hypergammaglobulinemia, numerous autoantibodies, and an increased prevalence of non-Hodgkin's lymphoma, suggesting a possible role for the B cell and the antibodies they produce in the mechanism of secretory dysfunction.

The cumulative data from the studies in the various congenic strains of the NOD mouse model of SS has redefined the pathogenesis of autoimmune exocrinopathy. As models of SS, these mouse strains have played an instrumental role in the elucidation of

the underlying events occurring in the initiating and progressive stages of the human disease. The characterization of the NOD-*scid* mouse, a congenic derivative of the NOD mouse, showed conclusively that the immune system was responsible for the loss of glandular function, in that the genetic ablation of the lymphocyte populations prevented gland hypofunction. However, this animal model also revealed that the loss of acini in the salivary glands occurred independent of lymphocytic infiltration, as these animals evidenced morphological restructuring and elevated levels of apoptosis consistent with the NOD parental strain. Based on the observations in this mouse, a paradigm for disease development was proposed where the autoimmune attack on the exocrine glands was precipitated by the combination of an intrinsic glandular defect leading to morphological changes, and an immunological defect that predisposed the immune system towards a persistent and debilitating attack on these tissues. These studies eliminated the possibility that the loss of function was a result of an underlying glandular defect, but they did not reveal the nature of the immune response critical to the secretory incapacitation. Furthermore, these observations indicated a high level of PCD in the glands in the absence of the adaptive immune response, but did not rule out the potential that the immune-mediated secretory hypofunction could be the result of the induction of further apoptotic cell death by the immune system.

The dissection of the immunologic pathogenesis of SS has been bolstered by the creation of several additional congenic mouse strains. The contributions that the NOD.Igμ^{null}, NOD.IL-$4^{-/-}$, NOD.IFN-$\gamma^{-/-}$, and NOD.IL$10^{-/-}$ have made have significantly increased our understanding of the immunological attack on the salivary and lachrymal glands. Chapter 2 showed that, with the lack of B cells in the NOD.Igμ^{null} mouse, animals

maintained normal function and exhibited limited infiltration of the salivary and lachrymal glands. Thus, this mouse identified the B cells as the responsible population driving secretory suppression. Passive transfer studies of SS IgG into these mice reconstituted the physiological pathology, further isolating antibodies as the mediators of oral and ocular dryness in SS. These animals also exhibited elevated levels of apoptosis in the salivary glands consistent with measurements in the NOD mouse, suggesting that secretory dysfunction was truly an independent occurrence. However, as these animals also showed only limited infiltration of the exocrine glands, the inflammatory aspects of the autoimmune progression could not be entirely overlooked as a contributor to the decrease in gland function.

The genetic development of the NOD.IL-4$^{-/-}$, NOD.IFN-$\gamma^{-/-}$, and NOD.IL10$^{-/-}$ mouse strains have allowed us to more directly probe the relevance of the inflammatory and humoral phases of the autoimmune response in search of the mechanism of secretory dysfunction. In theory, the lack of IL-4 and IL-10 would result in an exacerbated inflammatory response while the lack of IFN-γ would promote a shift towards a more dominant humoral response. Thus, we could explore the impact of each of these arms of adaptive immunity in the disease pathogenesis. The initial characterization of these mouse strains suggested that the lack of IL-10 did not significantly influence the onset of the disease, whereas the lack of either IL-4 or IFN-γ exerted profound influence on the pathological development. Therefore, we focused further attention on the latter two strains.

In terms of the dissection of the mechanism behind the loss of function, the IL-4-deficient NOD mouse proved to be the most informative. The NOD.IL-4$^{-/-}$ mouse

exhibited much more severe inflammation, as would have been predicted, and additionally, the focal infiltration developed earlier in the life of the animal. Despite the exacerbated focal infiltration, apoptotic activity remained consistent with that found in the NOD strain, suggesting that leukocytic infiltration and activation of PCD in the glands were separate manifestations of the disease. Another interesting observation in this strain was the development of anti-nuclear antibodies (ANA) despite the retention of normal secretory capacity throughout the animal's life. Thus, the NOD.IL-4$^{-/-}$ mouse provides strong evidence to distinguish focal infiltration and inflammation as a separate pathological entity from both elevated PCD and secretory suppression in this murine autoimmune exocrinopathy; and furthermore, PCD and secretory suppression can be distinguishes as two separate pathological entities themselves. Similarly, ANA can be ignored as an antibody mediator of the pathophysiology.

The NOD.IL-4$^{-/-}$ additionally provides us with several clues pertaining to the aspects of disease pathogenesis leading to the loss of glandular function in the later stages of the disease. The characterization of the NOD.Igμ^{null} mouse revealed that the B cell was critical for the development of secretory dysfunction, and passive transfer studies additionally identified the importance of autoantibodies in the mechanism of action. In that the absence of IL-4 does not alter the proportion of B cells infiltrating the salivary glands, the disturbance in B cell development in the SS-like pathogenesis of the NOD.IL-4$^{-/-}$ mouse occur after B cells have infiltrated the glands, but before they can mature to begin producing autoantibodies driving the glandular hypofunction. One significant difference in B cell development between the NOD parental strain and the NOD.IL-4$^{-/-}$

mouse is the lack of IgG1 production in the absence of IL-4. This suggests that the antibodies responsible for the loss of glandular function are of the IgG1 subclass.

The NOD.IFN-$\gamma^{-/-}$ mouse, like the IL-4 deficient partner strain, maintained normal salivary secretory capacities. Histological examination of the salivary glands revealed a complete absence of focal infiltrates until approximately 30 weeks of age and, equally interesting, normal acinar structure in the glands. Additionally, serological evaluation showed no signs for the presence of ANA. Thus, with respect to the mechanisms driving the decreased exocrine secretory rates, INF-γ appears to be essential to the pathogenesis of SS on a more global level and as such, it is necessary for the functional inhibition of the glands. However, it can be concluded that this cytokine is not a direct mediator of functional suppression in that the presence of IFN-γ in the absence of IL-4 cannot bring about this outcome.

The picture derived from the collective data obtained from the characterizations of these 4 congenic partner strains of the NOD mouse strongly implicates SS autoantibodies as the direct mediators of secretory hypofunction. Furthermore, these antibodies do not appear to be affecting this functional inhibition by inducing excessive apoptosis in the acinar cell population, thus killing off the secretory cells. However, since the exocrine glands of the NOD mouse appear to develop elevated levels of PCD in association with the presence of IFN-γ, it was necessary to look more directly at signaling pathways associated with SS autoantibody interactions in acinar cells. In an *in vitro* cell culture system using the submandibular acinar cell line SMG C6, IgG isolated from SS patient serum and specific M_3R-reactive monoclononal antibodies (developed by generating hybridomas from the fusion of NOD mouse B cells with SP2/0 cells) were tested for their

ability to induce caspase-3 activity as a reflection of apoptotic activity. In confirmation of the mouse studies, none of these antibody samples were able to generate significant caspase-3 activity at molar concentrations equivalent to physiologically active levels based on both competitive inhibition studies and passive transfer experiments. M_3R monoclonals, but not isotype control antibodies passively transferred into mice were able to block AQP translocation, though, suggesting that the mechanism of functional suppression of the salivary and lachrymal glands in SS relies on the interference with water channel formation at the acinar cell apical surface.

The concept of antibody-mediated interference of muscarinic receptor signaling resulting in secretory dysfunction is certainly an interesting possibility. There are numerous examples of immune recognition of neuroendocrine receptors, most obviously the nicotinic acetylcholine receptor in Myasthenia gravis[163] or thyroid hormone receptor recognition associated with Hashimoto's Thyroiditis and Grave's disease.[164,165] It is interesting to speculate as to why the immune system would specifically target these receptors. Recently, it has been suggested that autoimmune recognition could potentially have beneficial effects. Moalem, *et al.* (1999) reported that CD4+ T cells recognizing myelin basic protein, a classic autoantigen in multiple sclerosis, aids in the regeneration of injured optic nerves by effectively shutting down the neuron and thus preventing secondary destruction of the nerve fiber.[166] The antibody response against the M_3R in SS could be a similar manifestation, such that the inhibition of muscarinic M_3R signaling could be the attempts by the immune system to impose a functional quiescence in the salivary and lachrymal glands. The purpose of this "time-out" could potentially be to reduce the demands on the exocrine glands to promote the more efficient resolution of the

inflammatory response. This would help to explain the classical waxing and waning of symptoms experienced by SS patients. In support of this theory, current and ongoing research by Humphreys-Beher, *et al.* (unpublished data) demonstrates that NOD mice maintained on a liquid diet, thus decreasing salivary gland activity and saliva production, do not develop focal infiltration of the salivary glands or lose secretory capacity. It remains to be seen whether focal infiltration, immune-mediated glandular destruction and loss of secretory response can be reversed by the implementation of a liquid diet in mice exhibiting signs of the disease – or, for that matter, in SS patients. Regardless, this alternative perspective on autoimmunity presents an intriguing new perspective on the development of the anti-M_3R response and bring into question the strategy of treating SS patients with medications such as pilocarpine which increases saliva production by overstimulating the muscarinic response.

Clinical Implications

In terms of the SS patient, the conclusions drawn from this research have several implications. As previously, mentioned, many SS patients treat symptoms of oral dryness with pilocarpine tablets. Pilocarpine, a muscarinic agonist, forces the salivary glands to secrete saliva by hyperstimulating the M_3R. In light of the fact that the mechanism of secretory dysfunction in SS may be dependent on an antibody interaction blocking, downregulating or otherwise interfering with the M_3R, pilocarpine may actually exacerbate the situation over time. In effect, pilocarpine treatment will probably lead to the further downregulation of the M_3R from the cell surface, eventually resulting in increased secretory dysfunction. Thus, it may be time to reevaluate the clinical strategies for treating oral dryness in SS patients.

One of the most significant challenges in combating autoimmunity is to develop strategies to interfere with disease progression, and not simply treat the symptoms. While this is easier said than done, one concept that has captured a lot of attention has been immune modulation. Currently, patients with autoimmune diseases, including SS, are provided with immunosuppressants in more extreme cases to attempt to control the immune response. The importance of the T_H1 versus the T_H2 responses have been recognized for years, and attempts to classify specific autoimmune diseases as a T_H1- or T_H2-mediated process in hopes of being able to shift the course of the developing immune response to the opposite (and ideally less harmful) pathway. As we begin to understand the detailed progression of events that lead to the overt or late stage manifestations, we will hopefully be able to design more appropriate therapeutic protocols that target specific pressure points in the development of the autoimmune attack. Along these lines, IFN-α is currently being explored as a remedy for oral dryness in SS patients. Based on the studies in the cytokine deficient NOD strains, this protocol may be competing with IFN-γ as a critical immuno-regulatory factor. Just as in the NOD.IFN-$\gamma^{-/-}$ mice, antagonizing the effects of IFN-γ appears to reduce the impact of the immunological attack on the salivary secretory response.

And finally, these studies contribute to establishing the foundations for future work leading to the absolute identification of the antibodies responsible for secretory dysfunction. While the muscarinic receptor is a leading candidate as the functionally disruptive autoantibody, it may not be acting alone. The identification of the specific autoantibodies will ideally lead to the development of more stringent and reliable diagnostic tools in identifying SS than are currently available. Currently, aside from curing

the disease, the most formidable challenge in helping SS patients can be diagnosing this chronic disorder.

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BIOGRAPHICAL SKETCH

Jason Benjamin Brayer was born September 6,1970, in Hartford, CT. The son of Faith and Richard Brayer, he lived his entire childhood in Simsbury, CT. For his undergraduate education, he attended Bates College, in Lewiston, Maine, and received his B.S. in biology in 1992. In 1995, he accepted a position in the graduate research program at the University of Florida in the Department of Pathology and Laboratory Medicine. Under the co-mentorship of Dr. Michael G. Humphreys-Beher and Dr. Ammon B. Peck, he changed his departmental affiliation to the Department of Oral Biology and began his studies in the immunopathogenesis of Sjogren's syndrome.

To date, he has authored one article and has been included as coauthor on a number of other publications. Additionally, he has presented his studies at several conferences, including the 6th International Symposium on Sjogren's Syndrome and the 27th Annual Meeting of the International Association of Dental Research. His interest in immunology will take him next to the La Jolla Institute of Allergy and Immunology, where he plans to continue his work in autoimmunity and explore the regulatory events of the immune response that define autoimmunity from beneficial autoreactivity, such as in tumor immunity.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Michael Humphreys-Beher, Chair
Professor of Oral Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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